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## Introduction

About 5-10% of neurofibromatosis type 1 (NF1) patients are heterozygous for a contiguous gene deletion that includes the entire *NF1* gene. Although limited in scope, previous studies provide compelling evidence that microdeletion patients show early onset and large numbers of cutaneous neurofibromas, and a higher frequency of plexiform neurofibromas, malignant peripheral nerve sheath tumors, and other solid tissue malignancies. We propose to perform systematic, comprehensive clinical and molecular studies of subjects with *NF1* microdeletion to examine the gene(s) responsible for the severe tumor phenotype of microdeletion patients. The specific aims of this research are 1) To determine the clinical spectrum, genotype/phenotype correlations associated with heterozygosity for an *NF1* microdeletion. Genomic DNA of NF1 subjects will be examined by a multi-step screening protocol to identify germline microdeletion carriers, to map the extent of each deletion. We will correlate molecular data with the results of a comprehensive clinical evaluation of deletion and nondeletional NF1 control subjects. 2) To determine if cutaneous neurofibromas of germline *NF1* microdeletion patients show evidence of genomic instability or homozygous *NF1* microdeletion that may contribute to the early onset of neurofibromagenesis. Primary neurofibroma tissue from microdeletion patients will be analyzed to determine the presence and nature of 2nd hit mutations and whether these cells exhibit characteristics of genomic instability. 3) To screen candidate modifier genes in the *NF1* microdeletion region for mutations in subjects with early onset cutaneous neurofibromas who are not carriers of an *NF1* microdeletion. 4) To employ the newly developed FLASH technology to interrogate the *NF1* microdeletion region and construct a physical map that will determine, the sequence of all of the genes, unique noncoding regions, and paralogs (including the putative *NF1* duplicated gene) of the *NF1* microdeletion region.

## Body

**The original STATEMENT OF WORK and progress to date described below.**

### **Year 1, Months 1-8 Statement of Work in original grant application (underlined) and progress:**

- Develop a clinical database, train personnel to use. Completed and described in previous progress reports.
- Design and test clinic evaluation forms for patient assessment. Completed and described in previous progress reports.
- Design STS primers for interrogation of fosmid library.

This is part of aim #4 (see Introduction) to employ the newly developed FLASH technology to interrogate the *NF1* microdeletion region and construct a physical map that will determine, the sequence of all of the genes, unique noncoding regions, and paralogs (including the putative *NF1* duplicated gene) of the *NF1* microdeletion region. This importance was of critical importance due to a 2002 report claiming a tandem duplication of the *NF1* gene region based on fluorescence in situ hybridization (FISH) of stretched chromosomes and DNA fibers (1). Tandem duplication of the region would have significant impact on many aspects of NF1 research, e.g., mutational analysis, genotype/phenotype, and NF1 modifier studies, to name a few. Compelling evidence against an *NF1* tandem duplication was published in 2003 employing interphase FISH and an *NF1* translocation chromosome (2, 3). However, our collaborators at the University of Washington Genome Center sought to analyze genomic sequence data

for evidence of an *NFI* tandem duplication at 17q11.2 to assess the need and importance of aim #4, which was to construct whole genome fosmid libraries in several normal individual, construct a fosmid end map, and sequence the region to determine if there was genomic evidence in support of an *NFI* duplication.

**Conclusion: Our detailed analyses of 4 different data sources of genomic sequences (data shown below) did not support the presence of tandem *NFI* duplications at 17q11.2. Therefore, there is no valid scientific basis for pursuing aim #4 (a project that was to be supported by the University of Washington Genome Center and performed at no cost to this grant).**

#### **Assessment of validity of aim #4 using new genomic data:**

Drs. Marcia Paddock and Donald Bovee, University of Washington Genome Center, looked for evidence of a duplication of the *NFI* region on human chromosome 17 from four data sources: 1) SNP (single nucleotide polymorphism) reads from the SNP consortium, 2) Eichler's Human Segmental Duplication database (<http://humanparalogy.gs.washington.edu/>), 3) whole human genome shotgun reads from the non-public human genome sequence assembled by Celera (4) and 4) whole chimp shotgun reads from Washington University. The Human Segmental Duplication database and data on the location of the SNP consortium reads both use coordinates from build 30 of the human genome (June, 2002).

If *NFI* was duplicated in large part on chromosome 17, but then mis-assembled such that there appeared to be only one *NFI*-like region, one might find a greater than expected read density for the *NFI* region.

1). Results of SNP analysis: Using a 100KB window on chromosome 17, SNP read densities were examined across the chromosome. Excluding one 100KB window that yielded in excess of 12000 reads, I found an average read density of 134.4 and standard deviation of 48.7. The read densities for the two 100KB windows covered the *NFI* region were 101 and 61. The lower than average SNP read density would be consistent with a single, conserved *NFI*-gene containing area. Below is a detail from the area surrounding the *NFI* region. The yellow highlight indicates the *NFI* region. (The number on the left indicates the larger of the two bounds in kilo basepairs, i.e. '7' indicates the region between 600,000 and 700,000 of the named contig.)

10	NT_024897	122
1	NT_035420	89
2	NT_035420	104
3	NT_035420	88
4	NT_035420	138
5	NT_035420	124
6	NT_035420	130
7	NT_035420	204
8	NT_035420	154
9	NT_035420	101
10	NT_035420	61
11	NT_035420	143
12	NT_035420	109
1	NT_010799	71
2	NT_010799	98
3	NT_010799	139

4	NT_010799	98
5	NT_010799	111

2). Results of Eichler's Human Segmental Duplication database analysis: The Eichler database did not show any duplications of any part of the *NFI* region on chromosome 17, though there are many duplications of parts of the *NFI* region on other chromosomes (fragments of *NFI* pseudogenes). The two yellow highlighted lines are before and after where the *NFI* region sits on chromosome 17 using build 30. Read lengths do not support duplication of *NFI* region.

chrom	chromStrt	chromEnd	Other	O-Start	O-End	uid	length
chr17	29008187	29010469	chr17	62469756	62472220	14312	2464
chr17	29016354	29018320	chr17	43183961	43185786	14313	1825
chr17	29020061	29023975	chr17	62451103	62454756	14314	3653
chr17	29021424	29023976	chr17	28936356	28938947	14279	2591
chr17	29021424	29023976	chr17	30188164	30190754	14315	2590
chr17	29912188	29917543	chr17	25375319	25380000	14205	4681
chr17	29933752	29935087	chr17	29002934	29004257	14308	1323
chr17	29933752	29940753	chr17	65946336	65952615	14316	6279
chr17	29936847	29947955	chr17	65520869	65531978	14317	11109
chr17	29936847	29947955	chr17	44594051	44605152	14318	11101

3) Results of analyzing whole human genome shotgun sequence reads from the non-public human genome sequence assembled by Celera. Blasting the *NFI* region and ten other regions 200KB long that are also on chromosome 17 against Celera reads (masked) shows an increased read density for the *NFI* region, but when the number of reads is adjusted for duplicated pseudogene fragments (using Eichler's data) of *NFI* that exist elsewhere in the human genome, the number of reads for *NFI* is near the average.

Sequence read densities from sources			
Fasta file	Celera	Celera Adjusted	Chimp
<i>NFI</i>	1319	842	284
NT_010641_200-400	676	676	276
NT_010641_800-1000	759	759	288
NT_010718_800-1000	786	786	273
NT_010748_800-1000	1118	1038	273
NT_030843_200-400	2220	2180	293
NT_030843_800-1000	954	871	306
NT_078100_200-400	994	994	286
NT_078100_800-1000	666	666	291
NT_035425_200-1000	765	765	322
NT_035425_800-1000	572	572	304

These read densities were calculated blasting masked reads against the masked human

fragment database at Celera. Read densities change when the cutoff value (E value) for acceptance is changed from  $E=0.0001$  (most stringent allowed through the Celera interface) to something less stringent. Using the adjusted Celera data above, the average is 932 and standard deviation is 439. Excluding the single high value of 2180, the average is 797 and the standard deviation is 145.

4) Results of analyzing whole human genome shotgun sequence reads from the chimp genome. As shown in the table above, chimpanzee whole shotgun reads show no clear sign of increased read depth for the *NF1* region.

**Conclusion:** Our detailed analyses of 4 different data sources of genomic sequences (data shown below) did not support the presence of tandem *NF1* duplications at 17q11.2. Therefore, there is no valid scientific basis for pursuing aim #4 (a project that was to be supported by the University of Washington Genome Center and performed at no cost to this grant).

#### **Years 1-2 Statement of Work in original grant application (underlined) and progress:**

- Enroll new patients in the study. Enrollment of patients was significantly delayed by the time required for the Army HSRRB to approve our application (submitted July 2003, approved February, 2004) and has remained a delaying factor in our research. We currently have enrolled 43 subjects. These are in addition to existing DNAs from other previously ascertained patients and anonymous DNAs from our collaborator Dr. Mautner in Hamburg.
- Screen for *NF1* microdeletion patients, map extent of deletions, develop new deletion junction assays as needed. Three types of assays are described below:

1) PCR-based assays to detect the recurrent 1.4 Mb deletions at PRS1 and PRS2 recombination hotspots.

*NF1* germline microdeletions occur by non-allelic homologous recombination between NF1REP-P1 and NF1REP-M, which are 51 kb direct repeats that share 97.5% sequence identity (5-7). We have previously shown that these deletions map to two distinct paralogous recombination hotspots within the NF1REP sequences, which are denoted PRS1 and PRS2 (paralogous recombination sequences) (6-8). During this grant period, we developed two deletion-specific PCR assays that each generated a 7 kb product only if a deletion is present that was generated by recombination between either the two PRS1 or two PRS2 sites in NF1REP-P1 and NF1REP-M. We employed these assays to screen known *NF1* deletion patients from a number of our collaborators to determine the frequency of deletion breakpoints at these locations (see results in table below). These data show that these two assays rapidly detect the majority (~70%) of NF1-REP-mediated microdeletions. Therefore these data and the assays we developed can be used for rapid detection and diagnosis of *NF1* microdeletion carriers in either the research or clinical setting.

A major goal for the coming year is to rewrite this manuscript for publication. A manuscript of these data was originally submitted concurrently (to separate journals) with the Forbes et al (6) manuscript. These manuscripts “crossed” in the mail, with one accepted and one rejected at their respective journals. Forbes et al. was accepted and published; however, because it summarized some of the data in this manuscript, we must now re-package these data such that they

complement and do not repeat the data described by Forbes et al. I am currently re-writing this manuscript with a focus on diagnosis of *NF1* microdeletion and will submit it to a journal with a molecular diagnostic focus.

*NF1* microdeletion cases detected by PRS deletion junction assays.

Deletion Junction	Assay		Junction fragment sequenced (# cases)
	Shorter amplicon (3-3.4 kb)	Extended amplicon (7 kb)	
PRS1	13	14	14
PRS2	33	40	29
Total	46/78	54/78	

Data from Michael O. Dorschner, Hilde Brems, Rosalynda Le, Thomas De Raedt, Margaret R. Wallace, Cynthia J. Curry, Arthur S. Aylsworth, Eric A. Haan, Elaine H. Zackai, Conxi Lazaro, Ludwine Messiaen, Eric Legius, Karen Stephens

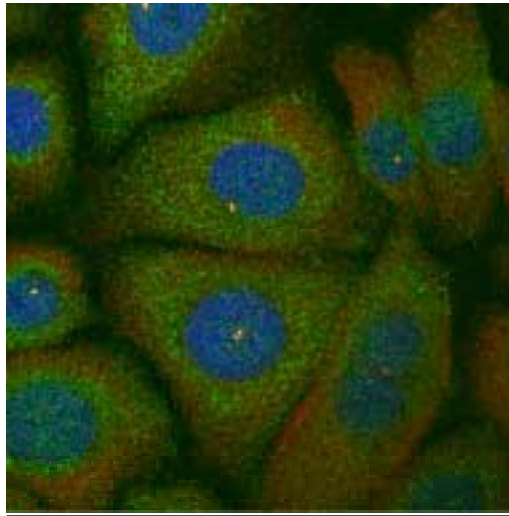
2) Assays to detect microdeletions that did not occur at PRS1 or PRS2. These assays employed real-time competitive PCR to measure gene dosage. In last year's progress report we gave an extensive and detailed description of a series of new quantitative PCR (qPCR) assays at 5 loci that we developed to detect *NF1* regional deletions. As detailed last year, every aspect of these assays was sound: reproducibility and low intra- and inter-assay variation on bona fide disomic and monosomic *NF1* patients. Importantly, the approximated 95% confidence limits (measured as  $\pm 2$  S.D.) for the range of normal and deleted values did not overlap. Inter-assay variations at each locus demonstrated distinct value ranges for normal and deleted (i.e., disomy and monosomy). The assays were developed for four loci within the region, AH1 (proximal to *NF1*), *NF1* exon 5, *NF1* exon 49, and WI-9521 (distal to *NF1*). The flanking markers AH1 and WI9521 are located within the common PRS1/PRS2-mediated microdeletions. In addition a gene dosage assay was developed for a control locus D17S250, which is distal and never observed to be co-deleted along with *NF1*.

Since last year we have extended screening patients by these assays and have performed experiments to confirm the putative deletions detected by these qPCR assays. Our results have been perplexing and disappointing with regard to these assays. These assays detected 16/198 (8%) of patients screened with putative deletions involving part or all of the *NF1* gene and flanking loci AH1 and WI9521. We employed FISH and/or polymorphic marker analysis (including family members when available) to confirm these data and surprisingly found that 12/16 were not deleted (nor were mosaic for deletion). So what was the problem? We have determined that some archival DNA samples of patients apparently had inhibitory substances that interfered with the qPCR; when fresh DNA prepared from the same lymphoblasts used for the FISH studies was used in the same qPCR assays, there was no evidence for deletion. Therefore, while the assays are precise, their use for screening archival DNA samples has reduced sensitivity. Of the remaining 4 putative deletion cases, one was FISH-confirmed mosaic for an *NF1* deletion, 2 others are marker-confirmed and awaiting FISH studies, and the last is a probable mosaic. Therefore, we developed and validated qPCR assays that identified 4 likely *NF1* microdeletions with breakpoints outside of the NF1REP paralog regions. Several FISH experiments are needed to complete this work for publication.

The qPCR assays we employed are based on standard curves and melting curve analysis (as

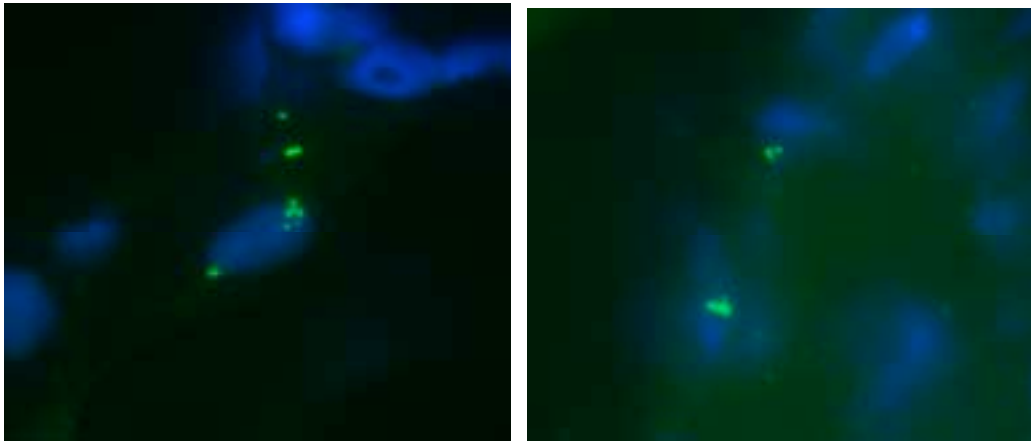
summarized below). Because the results gave some false positives with some archival samples, I plan to reanalyze the data using the delta  $C_t$  method to determine if that is a more robust method of analysis. Taken together, these new assays were more cumbersome and less sensitive than anticipated. Method: In brief, the qPCR assays are adapted from a method by Ruiz-Ponte et al (9) in which a known copy number of a competitor is introduced directly in the PCR along with the target patient DNA. The competitor is a mutated PCR product that is synthesized and cloned. The competitor is amplified by the same primers as the target, but differs slightly in DNA composition such that it will melt at a different temperature. PCR of genomic and competitor DNAs are performed in a capillary in the LightCycler instrument, followed by a melting curve. Once the concentration of competitor is determined for a specific concentration of normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same concentration of target DNA. Prior to the competitive PCR assays, we determined the exact concentration of the genomic DNA of each patient to be screened using real-time quantitative PCR at TPA (tissue plasminogen activator, chromosome 12). The TPA gene is amplified in each patient and compared to a standard curve using normal control DNA. This is used to calculate exactly what volume of patient DNA must be added to the competitive PCR assay.

- Ascertain NF1 subjects that show early onset cutaneous neurofibromas that do not carry microdeletions. To date we have identified 5 such patients, including one family that appears to have early onset cutaneous neurofibromas. Assessment of additional patients is ongoing.
- Determine conditions for immunohistochemistry, test and choose optimal antibodies. *This work relates to Aim #2 - To determine if cutaneous neurofibromas of germline NF1 microdeletion patients show evidence of genomic instability.* While there are several levels of genomic instability that can be detected in cells, one is centrosome abnormalities, which lead to abnormal mitoses and consequent losses and gains of chromosomes. We propose that this might be a mechanism in NF1-related tumorigenesis because
  - 1) we have found that neurofibromin localizes to the centrosome in human primary keratinocytes (see figure below), several simple epithelial cell lines, mouse 3T3 fibroblasts (manuscript in preparation). Interestingly neurofibromin did not co-localized with the centrosome in normal cultured Schwann cells (complements of Dr. David Muir).



Confocal microscopy of primary human foreskin keratinocytes where blue is DAPI-stained nucleus, green is anti-neurofibromin, and red is anti-gamma tubulin (primary component of centrosomes). Yellow = centrosomes double stained with tubulin and neurofibromin. Data of David Pearton and Karen Stephens, unpublished.

- 2) Centrosome abnormalities in size, number, and structure are hallmarks of many solid and hematologic malignancies and pre-malignant neoplasms (10-13). We have confirmed that neurofibromin co-localizes to the abnormally structured and clumped multi-centrosomes known to be present in squamous cell carcinoma cell lines (manuscript in preparation). Experiments to investigate if neurofibromin co-localizes to normal and or abnormal centrosomes in primary Schwann cells cultured from an NF1-related MPNST and MPNST cell lines will be examined this summer. We also have a few frozen neurofibromas and MPNST where this question can be examined in primary tissues. Regardless of the localization of neurofibromin, these experiments will assess whether tumors NF1-related tumors exhibit a form of genomic instability characterized by abnormal numbers of centrosomes and abnormal multipolar mitoses. This will provide new insight into tumorigenesis and may provide a marker for the transition from benign to malignant neoplasia. Preliminary experiments staining a single NF1-related neurofibroma with anti-centrin (a centrosomal protein) showed abnormal numbers of centrosomes.



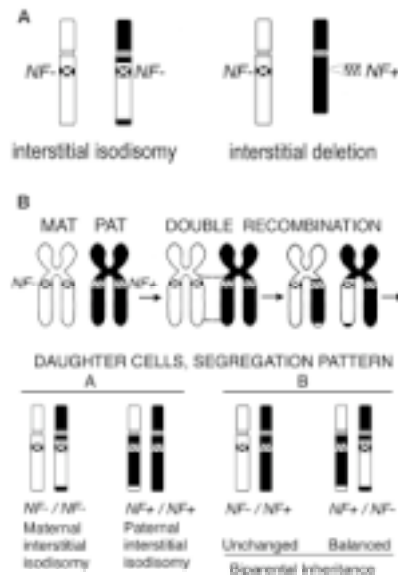
Immunofluorescence of an NF1-related neurofibroma showing a DAPI (blue) stained nucleus and a FITC (green) stained centrosomes, which are abnormal in number and arrangement. Note that the central cell in the left figure gives the appearance of being in early metaphase. If so, the centrosomes would be expected to lead to a multi-polar spindle and abnormal chromosome segregation.

- Related to our work on genomic instability in NF1 solid tumors, we have recently published our discovery of interstitial uniparental disomy as a novel mechanism of genomic instability during leukemogenesis in children with NF1. This research was initiated and performed in my laboratory, in collaboration with the laboratories of Drs. Keven Shannon, Michelle Le Beau, and Peter Emanuel, during the project period of my previous award from the CDMRP NF1 Program. Upon submission to two different genetics journals, we found that both wanted the experiments extended because the observations were so novel. Due to lack of patient samples, this was not possible, so our data were shelved. In the fall of 2005, cases of segmental uniparental disomy (not interstitial) involving the JAK2 locus were described in myeloproliferative disorders (14). Therefore, we updated our manuscript and submitted it to the journal *Blood* and it has been accepted and is in press. We can only assume that 4 years ago our findings were ahead of their time and difficult for some reviewers to believe. See Appendix for a preprint of Stephens K, Weaver M, Leppig KA, Maruyama K, Emanuel PD, Le Beau MM, Shannon KM. Interstitial uniparental isodisomy at clustered breakpoint intervals is a frequent mechanism of *NF1* inactivation in myeloid malignancies. *Blood*, in press. Although the great majority of the research was supported by a prior grant (DAMD17-1-7344), my effort to revive and update the data and submit the manuscript was supported by this present grant award.

- Abstract of article:

To identify the mechanism of loss of heterozygosity (LOH) and potential modifier gene(s), we investigated the molecular basis of somatic *NF1* inactivation in myeloid malignancies from ten children with neurofibromatosis type 1. Loci across a minimal 50 Mb region of primarily the long arm of chromosome 17 showed LOH in 8 cases, whereas a <9 Mb region of loci flanking *NF1* had LOH in the remaining 2 cases. Two complementary techniques, quantitative PCR and fluorescence in situ hybridization (FISH), were employed to determine if the copy number at loci that showed LOH was one or two (i.e., deleted or isodisomic). The 2 cases with LOH limited to <9 Mb were intrachromosomal deletions. Among the 8 leukemias with 50 Mb LOH segments, four had partial uniparental isodisomy and four had interstitial uniparental isodisomy. These isodisomic cases

showed clustering of the centromeric and telomeric LOH breakpoints. This suggests that the cases with interstitial uniparental isodisomy arose in a leukemia-initiating cell by double homologous recombination events at intervals of preferred mitotic recombination. Homozygous inactivation of *NF1* favored outgrowth of the leukemia-initiating cell. Our studies demonstrate that LOH analyses of loci distributed along the chromosomal length along with copy-number analysis can reveal novel mechanisms of LOH that may potentially identify regions harboring “cryptic” tumor suppressor or modifier genes whose inactivation contribute to tumorigenesis.



LOH in NF1-associated myeloid malignancies and proposed mechanism of interstitial isodisomy. The schematic in panel A depicts the two different patterns of LOH observed in the tumors. The inactivated *NF1* allele (NF-) is marked with an X on the chromosome, while the normal *NF1* allele (NF+) is indicated by diagonal hashmarks (///). The interstitial isodisomic and deleted regions can be of maternal or paternal in origin. Panel B. Proposed mechanism for double mitotic recombination during the S/G2 phase of the cell cycle leading to interstitial uniparental isodisomy in a leukemic-initiating cell. The four possible daughter cells are depicted, along with their *NF1* genotypes and disomy patterns. Although this example depicts a cell with maternal interstitial isodisomy and *NF1* inactivation, paternal interstitial isodisomy was also observed in our study.

## **Year 2 Statement of Work in original grant application (underlined) and progress:**

- Screen subjects with early onset cutaneous neurofibromas that are heterozygous at *NF1* for somatic mosaicism for an *NF1* microdeletion. The cells are ready and will be analyzed by FISH this year.
- Construct STS-content maps, sequence fosmids, and construct haplotypes. This is part of Aim #4, which our data described above indicate is not necessary to pursue.
- Obtain cutaneous neurofibromas from *NF1* microdeletion adults. To date we have obtained neurofibromas from two microdeletion patients, including multiple neurofibromas from on of the

patients. In addition, we have obtained MPNST from one of these patients. Samples are frozen in OCT. These will be examined by immunofluorescence for centrosome abnormalities.

- Perform immunohistochemistry and nucleic acid extraction of neurofibromas. This is in progress as described above.
- Assemble data on clinical spectrum of *NF1* microdeletion patients; write manuscript. Data is being assembled. We have not identified sufficient new deletion patients to date. Our collaborator, Dr. Mautner in Germany, has a large patient population and many *NF1* deletion patients.

### **Year 3 Statement of Work in original grant application (underlined) and progress:**

Note: Much of the work that we planned to have accomplished by the end of year 3 has been delayed due to pregnancy leave of Rosalynda Le, a talented technologist who has been in my lab for 15 years. She is returning May, 2006 and will focus on these experiments. We may request a no-cost extension to complete these studies.

- Screen *JJAZ1* gene for inactivating mutations in subjects with early onset cutaneous neurofibromas that are heterozygous at *NF1*. Will be performed during year 4.
- Continue to clinically evaluate age-matched *NF1* patients without microdeletions and control patients for comparison to determine prognostic utility of a microdeletion.
- Continue to obtain cutaneous neurofibromas from *NF1* microdeletion adults.
- Continue to perform immunohistochemistry and nucleic acid extraction of neurofibromas. In progress.
- Perform microsatellite instability studies on neurofibromas tissue. In progress for 5 tumors.
- Identify 2nd hit *NF1* mutations in neurofibroma. In progress for 5 tumors.
- Write manuscript. Manuscript on neurofibromin localization to centrosome of epithelial cells and squamous cell carcinoma is in progress.
- Continue fosmid analysis of *NF1* region; construct new libraries if needed. This is part of Aim #4, which our data described above indicate is not necessary to pursue.

### **Year 4 Statement of Work in original grant application (underlined) and progress:**

- Submit clinical information on *NF1* microdeletion patients to the National Neurofibromatosis Foundation International Database. Anticipated to contact Dr. Jan Friedman, director of the database this fall for submission of data.
- Analyze data for phenotype/genotype correlations and prognostic utility.
- Analyze the complete sequence of the *NF1* microdeletion region for new genes and paralogs. This is part of Aim #4, which our data described above indicate is not necessary to pursue.
- Perform comparative mapping of final human sequence with that of the mouse. This is part of Aim #4, which our data described above indicate is not necessary to pursue. Furthermore and this work has been completed and published by us and our collaborators (15).

### **Key Research Accomplishments**

- In collaboration with Drs. Marcia Paddock, Donald Bovee, and Maynard Olson of the University of Washington Genome Center, we sought genomic sequence data in favor of a tandem duplication of the *NF1* region on human chromosome 17 by analyzing SNP and

sequence read depths, which should be greater if a duplication exists. These data, replicated from several sources, supported the existence of only a single *NF1* gene at 17q11.2.

- Consistent with our hypothesis of genomic instability during NF1-related tumorigenesis, we have shown that loss of *NF1* heterozygosity in NF1-related leukemias occurs primarily by a novel mechanism of interstitial uniparental disomy with clustered breakpoints. The clustered breakpoints suggest chromosomal regions where mitotic recombination is favored along with selection for outgrowth of recombinants. Manuscript Stephens K et al., Interstitial uniparental isodisomy at clustered breakpoint intervals is a frequent mechanism of *NF1* inactivation in myeloid malignancies. Blood, in press.
- Consistent with our hypothesis of genomic instability during NF1-related tumorigenesis, we have demonstrated that neurofibromin localizes with the centrosome in normal primary cells and in cell lines. This suggests a possible role for neurofibromin normal centrosome regulation, at least in some cells. This discovery also poses the possibility that neurofibromin haploinsufficiency or deficiency in some tumor cell types may lead to centrosome dysregulation and abnormal mitosis. This hypothesis is supported by detection of neurofibromin in abnormal centrosomes of squamous cell carcinoma cell line and the preliminary detection of abnormal centrosomes in one NF1-related neurofibroma.
- We developed, validated a qPCR assay to screen for novel *NF1* microdeletions. This assay identified 4 such cases of 198 screened. While the assay functions well, it is not as robust as anticipated, particularly with archival DNA specimens.
- We have obtained multiple neurofibromas from one *NF1* deletion patient and samples of MPNST from a second *NF1* microdeletion patient.
- We developed deletion-specific PCR assay for rapid and reliable detection of the 1/4Mb *NF1* microdeletions that occur at the common recombination hotspots, PRS1 and PRS2. These assays demonstrated that about 70% of germline deletions occur at these sites. This manuscript is being revised for re-submission.
- Published a review of *NF1* microdeletions in the first book to focus on genomic disorders. Stephens K. Neurofibromatosis 1. *In Genomic Disorders: The Genomic Basis of Disease*, Eds.. JR Lupski, PT Stankiewicz. New Jersey: Humana Press, pp. 207-219, 2006.
- Dr. Stephens has attracted a new scientist to the NF1 field. Consultation and mentoring of Dr. Melanie Kuechle, Assistant Professor of Dermatology, has led to new work by her that neurofibromin may be a caspase substrate. She has applied for funding for this project.

## Reportable Outcomes

### Manuscripts

Lombillo VA, Sybert VP. Mosaicism in cutaneous pigmentation. *Curr Opin Pediatr*.17:494-500, 2005  
(see Appendix for reprint)

- Siegel DH, Sybert VP. Mosaicism in genetic skin disorders.  
*Pediatr Dermatol.* 23:87-92, 2006 (see Appendix for reprint)
- Stephens K. Neurofibromatosis 1. *In Genomic Disorders: The Genomic Basis of Disease*, Eds.. JR Lupski, PT Stankiewicz. New Jersey: Humana Press, pp. 207-219, 2006. (see Appendix for reprint)
- Stephens K. Clinical Molecular Genetics of the Neurofibromatoses. In *Neurocutaneous syndromes in Children*, Eds., P Curatolo, D Riva. Mariani Foundation Paediatric Neurology Series, n.15. Montrouge: John Libbey Eurotext, 2006. (see Appendix for corrected proofs).
- Stephens K. Neurofibromatosis. *In Molecular Pathology in Clinical Practice*. D.G.B. Leonard, Ed. New York: Springer –Verlag, in press. (see Appendix for preprint).
- Stephens K, Weaver M, Leppig KA, Maruyama K, Emanuel PD, Le Beau MM, Shannon KM. Interstitial uniparental isodisomy at clustered breakpoint intervals is a frequent mechanism of *NF1* inactivation in myeloid malignancies. *Blood*, in press. (see Appendix for preprint).

### Abstracts

- De Raedt, Heyns I, Brems H, Stephens K, Marynen P, Legius E. On the origin of *NF1* microdeletions. The CTF International Consortium for the Molecular and Cell Biology of NF1, NF2 and Schwannomatosis, Aspen, June, 2005

### Presentations

- Virginia P. Sybert, “Molecular Genetics for Dermatologists” Massachusetts Academy of Dermatology, Woodstock, VT, September 2005.
- Karen Stephens, “How can new NF1 research help patients now and in the future?” Neurofibromatosis Symposium, Washington State Neurofibromatosis Families, Children’s Hospital and Regional Medical Center, Seattle, WA, October 8, 2005.
- Virginia P. Sybert, “Genetic Disorders of Pigmentation” and “Molecular Genetics for Dermatologists, Mayo Clinic Visiting Professor, Genome Series Talks, Rochester, MN, January 2006.
- Virginia P. Sybert, “Molecular Genetics for Dermatologists”, Grand Rounds, Div Derm, University of Washington, Seattle, WA, February 2006.
- Virginia P. Sybert, “Genetic disorders of Pigmentation”, Teaching Conference, U. of Minnesota Dept Dermatology, March, 2006.
- Karen Stephens, “Genomic Disorders and their Mechanisms”, graduate class PATH 516 Molecular Basis of Human Genetic Disease, University of Washington, Seattle, WA, April 5, 2006.
- Karen Stephens, “Nucleotide Expansion Disorders”, graduate class PATH 516 Molecular Basis of Human Genetic Disease, University of Washington, Seattle, WA, April 12, 2006.
- Virginia P. Sybert, “Dermatologic Markers of Genetic Disease”, CME for Pediatricians, Group Health Cooperative, Seattle, WA, May, 2006.
- Karen Stephens, “Genome duplications/deletions/ impact on disease”, graduate class PATH530 Human Cytogenetics, University of Washington, Seattle, WA, May 16, 2006.

### Grant Review Responsibilities

- Congressionally-Directed Medical Research Program, Neurofibromatosis  
 Investigator-Initiated Review Panel, Scientist Reviewer, April, 2006.

## Conclusions

We have developed rapid and sensitive assays for the detection and mapping of both the common 1.4 Mb *NF1* microdeletion and novel microdeletions. Clinical evaluation of these patients, along with age/sex matched NF1 patients that are not *NF1* deletion carriers, is in progress to delineate the clinical manifestations most prevalent in deletion patients. We have collected tumors from *NF1* microdeletion carriers, which are now being examined for mutations and genomic instability. We published a new mechanism of genomic instability for NF1-related leukemias that involves interstitial uniparental isodomy at clustered breakpoints in the ancestral leukemic cell. These data suggest somatic recombination is favored at specific chromosomal regions, which leads to neurofibromin-deficient tumor ancestral cells(s). Additional work on genomic instability of primary NF1-related neurofibromas has generated preliminary data that shows that centrosomes are abnormal in number and structure; although the type(s) of cells with these defects is unknown. These data, along with our data showing neurofibromin localizes to the centrosome in at least some primary cells and cell lines, suggests neurofibromin may have a normal function in centrosome regulation, which is disrupted during tumorigenesis. These observations are currently being studied in detail. We have analyzed both human and chimpanzee genome sequences in 4 different ways to determine if there is evidence that supports previous reports of a tandem *NF1* gene duplication at chromosome 17q11.2; all results were consistent with the presence of a single *NF1* gene copy. So what? We have made significant contributions to our understanding of germline and somatic rearrangements that contribute to NF1: Assays for detection of germline *NF1* microdeletions were developed. The novel mechanism of somatic *NF1* loss in NF1-related leukemias add to our understanding of leukemogenesis and identifies chromosomal regions where somatic recombination may be favored, which could have implications for tumorigenesis of solid neoplasms. The localization of neurofibromin to the centrosome in at least some cells, along with the observation that centrosomes appear abnormal in NF1-related neurofibromas, implicates neurofibromin in normal centrosome function and in maintaining genome stability. These observations will be critical to follow up in pathologically-relevant cells and in tumors.







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## Appendix

PDF files of the following manuscripts are attached to this report. To open these files, double click on the paper clip icon.

### Manuscripts

-  Lombillo VA, Sybert VP. Mosaicism in cutaneous pigmentation. *Curr Opin Pediatr*.17:494-500, 2005.
-  Siegel DH, Sybert VP. Mosaicism in genetic skin disorders. *Pediatr Dermatol*. 23:87-92, 2006.
-  Stephens K. Neurofibromatosis 1. *In Genomic Disorders: The Genomic Basis of Disease*, Eds.. JR Lupski, PT Stankiewicz. New Jersey: Humana Press, pp. 207-219, 2006.
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-  Stephens K, Weaver M, Leppig KA, Maruyama K, Emanuel PD, Le Beau MM, Shannon KM. Interstitial uniparental isodisomy at clustered breakpoint intervals is a frequent mechanism of *NFI* inactivation in myeloid malignancies. *Blood*, in press.

# Mosaicism in cutaneous pigmentation

Vivian A. Lombillo<sup>a</sup> and Virginia P. Sybert<sup>b,c</sup>

## Purpose of review

This article reviews the disorders of patterned dyspigmentation and discusses the pathogenesis of the pigmentary changes.

## Recent findings

A range of cytogenetic abnormalities has been detected in patterned pigmentary disease. This molecular heterogeneity correlates with the wide spectrum of clinical phenotypes observed. Many of the molecular defects overlap with genes known to play a role in pigmentation. Our understanding of the underlying genetic mechanisms for these mosaic conditions is evolving with advances in technology and dissection of the molecular pathways involved in melanocyte biology.

## Summary

The causal heterogeneity of patterned dyspigmentation promises to reveal clues about the differentiation, function, and control of melanocytes in embryonic and postnatal development.

## Keywords

hypomelanosis of Ito, lines of Blaschko, mosaicism, pigment disorders

## Introduction

Pigmentary mosaicism is a term that has been used to describe variegated patterns of pigmentation in the skin caused by genetic heterogeneity in the cells that make up the skin [1•]. It is a term we prefer not to use because it implies that the cause is two or more genetically or chromosomally abnormal cell populations within a single individual. This has not yet been proved to be the cause of all conditions in which one sees variegation of pigment; therefore, we prefer to use the more inclusive term 'patterned dyspigmentation,' reserving the use of 'mosaicism' to indicate genetic or chromosomal mosaicism. The molecular mechanisms that underlie these patterns are largely not understood, and there are likely to be many. In this review, we outline what is currently understood about the molecular and biochemical pathogenesis of patterned dyspigmentation.

## Genetics of pigmentation

Although many components besides melanin contribute to skin color (e.g., bilirubin, hemoglobin), in this review the term 'pigment' will be used to refer to those changes caused by melanin. Abnormal pigmentation can result from perturbations in any gene that participates in melanocyte development or function. Melanocyte biology is complex; numerous (>100) loci are associated with pigmentary defects in mice. Nearly as many genes have been described in humans; however, the list of human pigmentary genes is by no means complete. Several excellent reviews of the genetics of pigmentation have recently been published [2,3,4•]. Genes associated with pigmentation are broadly classified according to their role in melanocyte biology: (1) genes involved in the embryologic development of melanocytes (e.g., KIT in piebaldism and PAX3 in Waardenburg's syndrome), (2) genes contributing to melanosome formation in the melanocytes (e.g., the HPS genes in Hermansky-Pudlak syndrome and CHS1 in Chediak-Higashi syndrome), (3) genes responsible for melanin synthesis in the melanosome (e.g., TYR in oculocutaneous albinism 1), and (4) genes preserving melanosome transfer to neighboring keratinocytes (e.g., RAB27A and MYO5A in Griscelli's syndrome). Defects anywhere within this pathway may result in dyspigmentation. A partial list of genes known to be involved in disorders of pigmentation is shown in Table 1. A more comprehensive catalogue of the pigmentary genes has recently been compiled [1•].

## Mechanisms of mosaicism

A mosaic in the conventional sense is a composite of different materials. A mosaic organism is composed of

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## Abbreviations

HI	hypomelanosis of Ito
IP	incontinentia pigmenti
LWNH	linear and whorled nevus hypermelanosis
NFκB	nuclear factor kappa B

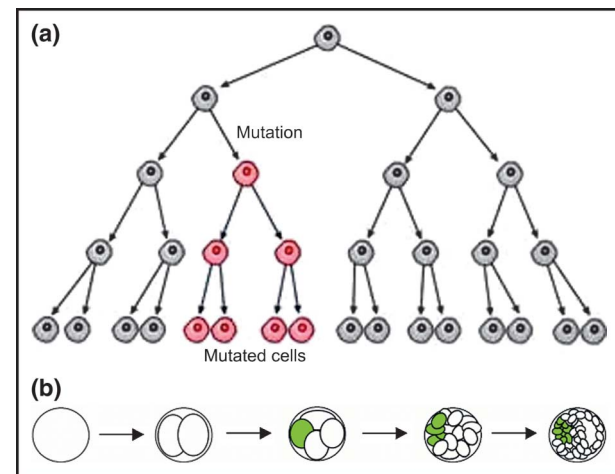
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**Table 1. Partial list of human skin disorders associated with dyspigmentation**

Disorder	Gene	Inheritance
Piebaldism	KIT	AD
Waardenburg syndrome (WS)		
WS1	PAX3	AD
WS2	MITF, SLUG	AD
WS3	PAX3	AD, AR
WS4	EDN3, EDNRB, SOX10	AD, AR
Oculocutaneous albinism		
OCA1	TYR	AR
OCA2	P	AR
OCA3	TYRP1	AR
OCA4	MATP	AR
Hermansky–Pudlak Syndrome (HPS)		
HPS1	HPS1	AR
HPS2	AP3B1	AR
HPS3	HPS3	AR
HPS4	HPS4	AR
HPS5	HPS5	AR
HPS6	HPS6	AR
HP7	DTNBP1	AR
Chediak–Higashi syndrome (CHS)	CHS1	AR
Grisceoli syndrome (GS)		
GS1	RAB27A	AR
GS2	MYO5A	AR
GS3	MLPH	AR
Incontinentia pigmenti	NEMO/IKBKG	XLD
Tuberous sclerosis	TSC1, TSC2	AD

AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant.

two or more genetically or chromosomally distinct populations of cells. A somatic or postzygotic mutation is a de-novo genetic change that occurs after fertilization, during embryogenesis, during fetal development, or during postnatal life. The stage of development in which the mutation or change occurs will determine which tissues and what proportion of cells within the tissue will contain the change. Only the tissue derived from the precursor cell in which the alteration has occurred is affected (Fig. 1a). Somatic mosaicism implies that two or more genetically or chromosomally distinct cell populations are present in the organism (Fig. 1b) and may or may not involve the gonad. If the gonad is not involved, there is no risk to offspring. Gonadal mosaicism implies that the tissue giving rise to the gametes is also mosaic and therefore carries with it the risk that gametes will contain genetic information from one or the other of the distinct populations and the risk that the mutation or chromosomal abnormality will be transmitted to offspring. Mosaicism always underlies the phenotypic variation of certain diseases, such as McCune–Albright syndrome (which is lethal in the fully heterozygous state) and explains the phenomenon of segmental disease (localized involvement) in conditions such as neurofibromatosis type 1, Darier's disease, and Hailey–Hailey disease [5,6,7<sup>•</sup>]. Mosaicism has also been implicated to contribute to malignant transformation in cancer

**Figure 1. The making of a mosaic organism**

(a) Demonstration of how a de-novo mutation generates a mutant population of cells after subsequent mitotic divisions. (b) An early mutation (green) at the four-cell stage generates a mosaic organism. Reproduced with permission by Dr. Carol Guze, PhD.

[8]. The phenomenon of mosaicism may occur by way of several mechanisms.

### Chromosome aneuploidy or polyploidy

Failure of chromosomes to separate properly during cell division can result in daughter cells with different or aberrant chromosome numbers or structures. Chromosomal mosaicism is common in Turner's syndrome; almost half of affected individuals have two or more chromosomally distinct cell populations. These may both be abnormal, as in 45,X/46,X,i(Xq), or one may be normal and the other not, as in 45,X/46,XX mosaicism. Some chromosomal aberrations are lethal when they are present in all tissues and are seen in live-born infants only in the mosaic state (e.g., tetrasomy 12p<sup>+</sup> or Pallister–Killian syndrome).

### Lyonization

In humans, to compensate for the presence of two X chromosomes (paternal and maternal) in females, one X chromosome in every cell of a developing female embryo is randomly but stably inactivated in the late blastocyst stage by way of alterations in chromatin structure and methylation states [9]. All females are therefore functionally mosaic with respect to most genes on their X chromosome. This process, also called lyonization, contributes to 'rescue' of otherwise lethal phenotypes in dominant X-linked conditions such as incontinentia pigmenti (IP) [10]. It is also responsible for the mosaic pattern of expression in carrier females of X-linked recessive conditions, such as hypohidrotic ectodermal dysplasia (Christ–Seimens–Touraine syndrome).

### Epigenetic mosaicism

There may be environmental mechanisms that can alter the genetic information within a cell that then gives rise to genetically distinct cell populations. One example is that of retrotransposons, DNA sequences of viral origin that are incorporated into the nuclear DNA and replicated. These may silence or activate gene expression [11]. These elements have been studied in relation to variegation of coat color in mice and dogs and have been shown to be heritable in mice [12–13]. Nothing is currently known about their role in human disorders of pigmentation.

Changes in chromatin structure due to chemical modification of histones (e.g., acetylation states) by encoded proteins also influence the phenotype of cells by altering gene expression [14<sup>\*</sup>]. Our understanding of the effects of chromatin structure on phenotypic variation in human disease is in its infancy, but this will be an important mechanism to consider. Lyonization and imprinting are specialized forms of epigenetic regulation of gene expression that are inherent and not environmental.

### Chimeric mosaicism

Although it is rare, some individuals with segmental or patterned dyspigmentation have been shown to be hematologically chimeric, composed of two genetically distinct cell populations [15]. This presumably results from an early fusion event of two zygotes or intra-uterine transfusion of cells between dizygotic twins. Chimeras may be 46,XX/46,XX or 46,XY/46,XY or 46,XX/46,XY. In the last, genital ambiguity and hermaphroditism is seen. The former situations presumably give rise to otherwise normal individuals. The distribution of pigment in these individuals is often in a geometric block pattern and not along the lines of Blaschko.

### Revertant mosaicism

A revertant mutation is a mutation that occurs focally and restores local function to a previously defective (inherited) gene. Although rare, this has been described in types of epidermolysis bullosa and tyrosinemia type I [16,17]. Whether this phenomenon occurs in single-gene disorders of pigmentation in which patchy repigmentation may occur (e.g., piebaldism) is not known.

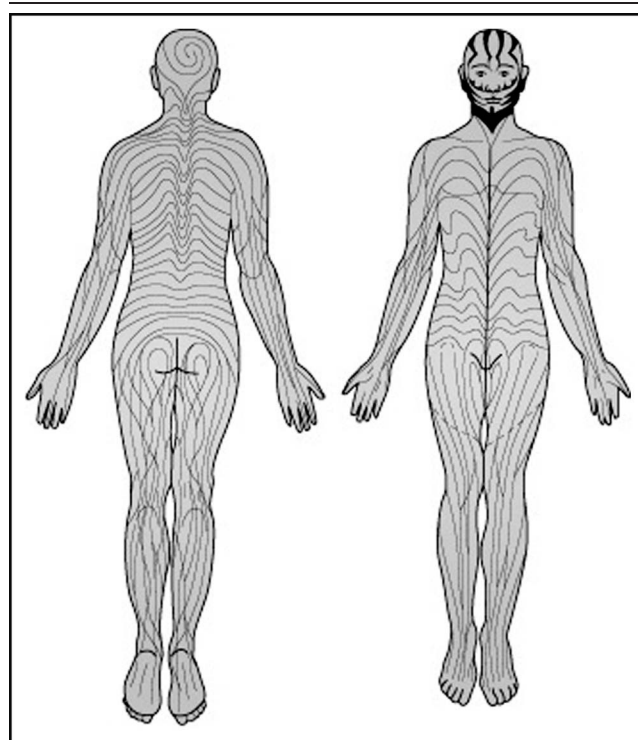
### Patterns of dyspigmentation: lines of Blaschko and its variations

Dermatoses resulting from mosaicism may show a classic distribution on the skin along the lines of Blaschko [18–20]. On the basis of the distribution of different nevoid and non-nevoid skin disorders, Blaschko [21] described a unique pattern of sharply demarcated lines and swirls, which is distinct from any other linear pattern on the skin,

such as dermatomes or Langer's lines (lines of cleavage). On the back there is a V-shaped or 'fountain spray' configuration, and on the abdomen the pattern is more S-shaped (Fig. 2). On the vertex of the scalp the lines are spiral. The extent and pattern of distribution in the skin is presumed to be dependent on the stage of development during which the event causing mosaicism took place. Very early events result in widespread variegation. Late events may cause changes to appear in only one sector, or even a small area within a sector. The patterns are seen in epidermal and sebaceous nevi, in the expression of X-linked conditions in carrier females, and in patterned dyspigmentation due to chromosomal mosaicism.

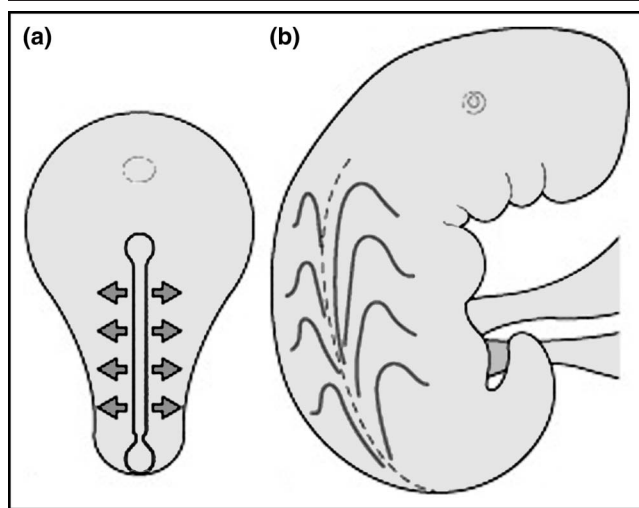
The lines of Blaschko are believed to map the route of embryonic ectodermal cell migration (Fig. 3) [19,22]. Both melanocytes and keratinocytes are derived from ectoderm. Whereas cytogenetic confirmation of tissue or cellular mosaicism for chromosomal abnormalities has been demonstrated in many patients with patterned dyspigmentation, failure to find it in all instances may be due to the fact that the mosaicism is not present in the cells that are usually studied, lymphocytes and fibroblasts, which are not of ectodermal origin. Somatic mutations have been demonstrated from keratinocytes isolated from

**Figure 2. Lines of Blaschko**



The developmental lines of Blaschko are distinct from any other linear pattern observed on the skin, such as dermatomes. Reproduced with permission from [20].

**Figure 3. Ectodermal migration and theory for the development of lines of Blaschko**



(a) Ectoderm-derived cells of the neural crest, including melanoblasts, migrate transversely along distinct dorsal-ventral pathways in the developing vertebrate embryo. (b) Happle has hypothesized that longitudinal growth and flexion of the embryo contributes to the fountain-like pattern known as Blaschko's lines. Reproduced with permission from [20].

epidermis in several cases of patterned skin disease [6,23,24].

Happle [25] describes several variations of the classic Blaschko pattern. A block-like or 'checkerboard' distribution has been described in speckled lentiginous nevus and Becker's nevi. The en-bloc or checkerboard pattern of pigment variegation is also typical of chimeras. A phylloid or 'leaflike' variant has been described for patients with mosaic hypopigmentation due to polyploidy or translocations involving chromosomes 13 or 5 [26–29].

### Clinical presentation of patterned dyspigmentation

The most common manifestations of patterned dyspigmentation are discussed in the next section.

#### Hypomelanosis of Ito

Hypomelanosis of Ito (HI), also called incontinentia pigmenti achromians, is a term used to describe distinct hypopigmentation in whorls and streaks that lie in the lines of Blaschko (Fig. 4a–c). Whereas the original description by Ito [30] was of a patient with patterned depigmentation, subsequent reports described individuals with hypopigmentation in the same pattern. The term 'HI' has also been applied to individuals with hyperpigmentation along the same lines. In some cases, it is not clear whether the normal skin is the darker or the lighter (e.g., Fig. 4a and c). The hypomelanosis may be present at birth, or it may become more apparent during early

childhood. HI affects both sexes equally and is considered sporadic. The distribution may be bilateral or unilateral and can involve any part of the body or face. The pigment variegation is most dramatic in individuals with darker skin and appears as a negative image of the hyperpigmented stage of IP. The textural skin changes of IP are not seen in HI. Chromosomal mosaicism has been reported in blood or skin fibroblasts of more than half of patients with fully evaluated HI [31]. Abnormalities have included chimerism; mosaicism for 2, 4p<sup>+</sup>, 7, 8, 9,10,13, 14, 15, and 17; ring X; tetrasomy 12p<sup>+</sup> (Pallister–Killian syndrome); X-autosome translocations; and diploid/triploid mosaicism. The specific chromosomes involved seem to be less important than the state of mosaicism for the abnormality. Mosaics for presumably balanced translocations in otherwise normal individuals can also give rise to the pigment pattern of HI. Systemic manifestations are seen in 30 to 90% of patients (depending on the study cited) and may involve the neurologic, ocular, cardiac, and musculoskeletal systems [32–34]. This heterogeneity is not surprising because it reflects the very different effects of different cytogenetic deletions or duplications.

Children with pigmentary changes along the lines of Blaschko should be examined for structural malformations and for developmental problems. In the absence of any phenotypic abnormalities, the karyotype is likely to be normal. In the presence of other major or minor malformations or developmental abnormalities, both lymphocyte and fibroblast karyotyping should be considered.

#### Nevus depigmentosus

Nevus depigmentosus (or achromic nevus) is a well-defined area of hypopigmentation or depigmentation, which is typically present at birth, although it may not be evident until the first years of life [35]. A single irregular ovoid patch (like a splash of paint) is commonly seen on the trunk and proximal extremities (Fig. 4d). Multiple patches and segmental forms along dermatomes or Blaschko developmental lines have also been described. Nevus depigmentosus is usually sporadic, with no gender predominance. By skin examination alone it may be difficult to differentiate the ash-leaf macule of tuberous sclerosis complex from a nevus depigmentosus. Nevus anemicus may also have a similar appearance. One can differentiate the two by rubbing the skin; a nevus depigmentosus will become more erythematous; however, a nevus anemicus will remain hypopigmented as the surrounding skin reddens. The underlying cause of nevus depigmentosus has not been identified. A case series of 67 patients with nevus depigmentosus found no associated abnormalities, and this is generally considered to be an isolated cutaneous finding [35]. A small handful of case reports, however, have described neurologic defects in children with nevus depigmentosus [33]. Two patients in a series

**Figure 4. Presentations of patterned dyspigmentation**

(a–c) Hypomelanosis of Ito. (d) Nervus depigmentosus. (e) Linear and whorled nevoid hypermelanosis. (f) Hyperpigmented stage of incontinentia pigmenti. Reprinted with permission from [34] (a–c,e,f) and [www.dermatlas.org](http://www.dermatlas.org) (Mary Tonsager) (d).

of 20 patients with a segmental type of nevus depigmentosus had mental retardation and seizures [36].

If systemic manifestations are not present, no further management is necessary for nevus depigmentosus. There is no effective therapy for repigmentation.

#### **Linear and whorled nevoid hypermelanosis**

Linear and whorled nevoid hypermelanosis (LWNH) is a usually sporadic disorder of hyperpigmentation in swirls

and streaks along the lines of Blaschko (Fig. 4e) [34]. This is also considered to be an isolated cutaneous condition. Both sexes are equally affected. The hyperpigmentation is seen in the first weeks of life and is not preceded by inflammatory stages, unlike IP. The pigmentation may expand for 1 or 2 years, but it stabilizes thereafter and may become less prominent in time. There have been reports of LWNH in association with central nervous system abnormalities and chromosomal mosaicism [33,37,38]. We prefer, however, to categorize these as HI, rather than

LWNH, and reserve the term LWNH for individuals who are otherwise normal.

### Incontinentia pigmenti

Incontinentia pigmenti is an X-linked dominant disorder, lethal in males, in which lethality in females is rescued by lyonization. Survival in males has been attributed to the presence of an extra X chromosome (Klinefelter's syndrome) or mosaicism for the gene because of post-zygotic mutations [39]. Infants with IP initially have blisters in lines and swirls along the lines of Blaschko that become erythematous and verrucous. Hyperpigmentation along the lines of Blaschko ensues (Fig. 4f). The fourth stage of IP occurs later and consists of hypopigmentation and loss of sweat glands and hair follicles in a similar Blaschko pattern. The early stages may occur *in utero*, and not all stages may be present. The blistering may recrudescence with illness in later childhood. Mutations in NF $\kappa$ B essential modulator (NEMO/I $\kappa$ BKG located on Xq28), a subunit of a kinase that activates NF $\kappa$ B, cause IP [40]. Failure to activate NF $\kappa$ B renders the affected tissue susceptible to apoptosis. In addition to the skin changes, ectodermal defects (e.g., pegged teeth, alopecia, and nail dystrophy), neurologic, and skeletal and ocular involvement may also be present.

### McCune-Albright syndrome

McCune-Albright syndrome (polyostotic fibrous dysplasia) is a disorder in which affected individuals are mosaic for mutations in GNAS1. Presumably, full heterozygosity for mutations in this gene is lethal, and mosaics survive because of the presence of a proportion of normal cells. It affects the skeleton, skin, and endocrine systems. Large 'coast of Maine' café-au-lait patches with midline demarcation characterize the pigmentary changes. Polyostotic fibrous dysplasia, hyperfunction of endocrine glands, and precocious puberty (almost exclusively in females) also occur. The café-au-lait patches are first noted in infancy. They are typically larger and have rougher edges than the café-au-lait macules of neurofibromatosis type 1; occasionally individuals with neurofibromatosis type 1 may have similar large café-au-lait patches. There may be distribution within Blaschko lines, although this is not always apparent [41]. The disorder is caused by missense mutations in GNAS1, a gene that encodes a G protein subunit (Gs- $\alpha$ ) that activates adenylate cyclase. This results in the production of excess endogenous cAMP in mutant cells [42]. How this causes cutaneous hyperpigmentation is not known; however, cAMP is known to play a role in melanogenesis and in melanosome transport [43,44,45•].

### Theories for patterned dyspigmentation

An insightful review of the literature by Taibjee and Moss [1•] revisits the pathomechanism for pigment variation in mosaic conditions. The authors raise the possibility of

a nonspecific border effect between two distinct cell lines. In other words, both the dark and light areas may be normal in melanocyte distribution and function, but different in the amounts of pigment produced. The apposition of the two different clones makes them clinically apparent. However, the authors favor the hypothesis that normal pigment production is disrupted because of the loss or gain of function of specific genes controlling pigment production. They systematically compared the locations of karyotypic abnormalities of 98 cases of patterned dyspigmentation with known loci for 76 pigmentation genes. 88% of the chromosomal abnormalities overlapped with the location of at least one known pigmentary gene. Conversely, three quarters of all known pigmentary genes are in regions that overlap with the chromosomal abnormalities found in cases of patterned dyspigmentation. Although this is not evidence for a causal relation of the disruption of specific genes involved in pigmentary changes, it is suggestive.

### Conclusion

We have not come much further in our understanding of the mechanisms leading to dyspigmentation in chromosomal mosaicism since the association of HI with chromosomal mosaicism was first recognized [46–48]. The role of cell–cell communication in the control of melanogenesis has not yet been investigated in HI. This may prove to be extremely interesting and may ultimately explain normal patterns of pigment distribution such as Futcher's lines. Why the single-gene disorders of Waardenburg's syndrome and piebaldism result in patterned dyspigmentation is also not understood. With the sequencing of the human genome and the rapid advances in genomic technologies, the next decade promises to greatly enhance our understanding of the molecular underpinnings of pigmentation.

### Acknowledgement

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## Mosaicism in Genetic Skin Disorders

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Mosaicism for genodermatoses often comes to the attention of pediatric dermatologists either because of patterned pigmentation or skin eruptions following the lines of Blaschko. For example, chromosomal mosaicism can result in linear and whorled pigmentary alteration. The same distribution pattern is seen in diseases resulting from the functional mosaicism in females for genes on the X chromosome including sweat patterns in carriers of X-linked hypohidrotic ectodermal dysplasia and females with incontinentia pigmenti. It is also found in the expression of X-linked disorders in males with Klinefelter syndrome, 47, XXY. Somatic mosaicism for single gene disorders explains the segmental distribution of many conditions (Table 1). In this column, we will review mechanisms and clinical examples of single gene, chromosomal, functional, and revertant mosaicism.

Mosaicism for single gene mutations results from events occurring after fertilization. This is referred to as somatic mosaicism. The earlier the mutational event occurs, the more likely it is that the individual will express the condition to a significant degree and the more likely it is that the gonadal tissue will be involved; thus conferring a risk of transmission to offspring. This is also called gonosomal mosaicism, meaning both the gonadal tissues and the somatic tissues are involved. If the mutation occurs after the cells that are committed to the gonad have formed, then the mosaicism will not involve the germline and reproductive risk is not an issue.

Gonosomal mosaicism is the mechanism for the occurrence of segmental neurofibromatosis (NF) in which affected individuals manifest the pigment changes and development of neurofibromas in a sector or quadrant or multiple, but noncontiguous areas, of the body, and

subsequently have children with full-blown NF1. Another example is perinatal lethal osteogenesis imperfecta (OI type II), a biochemically heterogeneous disorder, usually caused by new mutations in the genes for type I collagen (1). Somatic mosaicism with germline mosaicism (gonosomal mosaicism) in a mildly manifesting parent has been implicated as the cause for the occurrence of lethal type II osteogenesis imperfecta in offspring.

Gonosomal mosaicism was also demonstrated in 1994 when Paller et al (2) evaluated cells from individuals with epidermal nevi of the epidermolytic hyperkeratosis type who had offspring with generalized epidermolytic hyperkeratosis (EHK) for mutations in the keratin 1 (K1) and keratin 10 (K10) genes. They studied peripheral blood lymphocytes, and did skin biopsies from affected and unaffected skin to create keratinocyte and fibroblast clones. They found K10 point mutations in 50% of the alleles from cultured keratinocytes from epidermal nevi, whereas no mutations were detected in normal skin or blood from these parents. Their fully affected offspring were found to be heterozygous for the causal mutation in all cells examined.

As opposed to gonosomal, which involves both gonads and somatic tissue as described previously, mosaicism may also occur exclusively in gonadal tissue; this is referred to as gonadal mosaicism. Gonadal mosaicism has been implicated in the recurrences among siblings of autosomal dominant disorders such as achondroplasia, tuberous sclerosis, and neurofibromatosis in families where neither parent is apparently affected. This mechanism has been proved by examination of sperm from the fathers in some of these families, in which two genetically distinct cell populations can be found.

Two mechanisms for segmental mosaicism of autosomal dominant disorders were proposed by Happle. Type

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**TABLE 1.** *Mosaicism in Selected Genetic Skin Disorders*

Chromosomal	Autosomal dominant/single gene		Functional		
	Lethal	Nonlethal	X-linked dominant	X-linked recessive	Revertant focal correction
• Hypomelanosis of Ito	• McCune–Albright • CHILD	• NF1  • Tuberous sclerosis • Epidermolytic hyperkeratosis  • Darier • Hailey–Hailey	• Focal dermal hypoplasia • Incontinentia pigmenti • Conradi–Hunermann	• Hypohidrotic ectodermal dysplasia	• Dowling–Meara EB simplex • Recessive EB simplex • Non-Herlitz junctional EB

CHILD, congenital hemidysplasia with ichthyosiform erythroderma and limb defects.

1 presents with a segmental phenotype because of a somatic mutation, and the skin outside of the affected segment is normal, as is the genomic DNA (3). In type 2 segmental mosaicism, the affected individual has a heterozygous genomic mutation for an autosomal dominant condition but with exacerbation because of loss of heterozygosity within a segment or along the lines of Blaschko. This pattern has been described in individuals with several genodermatoses including superficial actinic porokeratosis with regions of overlying linear porokeratosis, Darier disease, tuberous sclerosis complex, and Hailey–Hailey disease. Hailey–Hailey disease is an autosomal dominant disorder caused by a mutation in the gene, *ATP2C1*, which encodes a calcium pump protein. Individuals with Hailey–Hailey present with red scaly plaques in the intertriginous areas of the body. Poblete-Gutierrez et al (4) described a patient with Hailey–Hailey disease who also had unilateral exacerbation with erythematous crusted plaques along the lines of Blaschko. Keratinocytes isolated from the classically affected areas were heterozygous for the mutation in *ATP2C1* as expected, whereas keratinocytes from the regions of linear exacerbation were homozygous for the mutation.

Segmental neurofibromatosis will now be reviewed in greater detail as a clinical example of nonlethal single-gene mosaicism. Segmental neurofibromatosis is a term that describes individuals who have cutaneous signs of neurofibromatosis limited to a defined area of the body. This may be either half of the body, a quadrant, or a smaller area defined by several lines of Blaschko. Skip or patchy involvement has also been described. Localized NF1 follows a similar natural history to generalized NF1 in the affected tissues. Café au lait patches and plexiform neurofibromas present early, whereas neurofibromas develop later in adolescence. Localized NF1 is unlikely to cause systemic complications (5).

Individuals with segmental NF1 are thought to develop their condition as the result of postzygotic mutations in the *NF1* gene. More extensive cutaneous

involvement is likely to be the result of an earlier mutational event and therefore carries a greater likelihood for involvement of the gonads and risk of transmission to offspring. Mosaicism caused by very late postzygotic mutations may be a mechanism by which isolated café au lait spots develop. Tinschert et al (6) recently published a study using fluorescence in situ hybridization on cultured fibroblasts from skin with a café au lait patch, and compared it with fibroblasts from normal-appearing skin from the same patient. They detected only a single hybridization signal in the affected skin, compared with two signals from clinically normal skin and lymphocytes, thereby demonstrating mosaicism for a deletion of the *NF1* gene in the fibroblasts from the café au lait patch (6). Consoli et al (7) described a parent with segmental NF who gave birth to a child with generalized NF1. They were able to demonstrate heterozygosity for a nonsense mutation in exon 31 of the *NF1* gene at variable levels in the affected skin from the mother, but not present in her peripheral blood lymphocytes. The mutation was present in the heterozygous state in both the fibroblasts and the peripheral blood lymphocytes of her child. What remains unexplained are several reports of families in which segmental NF1 appears to be segregating as an autosomal dominant condition with segmental expression in parent and child.

Individuals with autosomal dominant lethal conditions can sometimes survive as mosaics. McCune–Albright syndrome, which is characterized by large, segmental, unilateral, dark café au lait patches, polyostotic fibrous dysplasia, and endocrine abnormalities, is likely an autosomal dominant lethal condition in the heterozygous state. Happel (8) suggested that the individual can only survive when there is mosaicism for the mutation. It is now proved that McCune–Albright syndrome is caused by sporadic postzygotic mutations in *GNAS1* gene on chromosome 20q13.2 and that affected individuals are mosaic for these alterations (9). The severity and distribution of expression is dependent on the tissue distribution of the cells with the mutation. Individuals with

McCune–Albright are not at risk to have affected children, even if the germline is involved, because fertilization by a gamete with the *GNAS1* mutation will result in lethality of the conceptus.

*Chromosomal mosaicism* results from nondisjunction events that also occur after fertilization. This failure of the chromosomes to divide normally may lead to mosaicism for abnormal and normal cell populations, such as 45, X/46, XX Turner syndrome, or to mosaicism for two abnormal cell populations, such as 45, X/46, X, +ring(X) Turner syndrome. In the former situation, the conceptus was presumably chromosomally normal, with the failure of normal mitotic cell division subsequently; in the latter, the conceptus was abnormal and the small ring chromosome was presumably lost at some stage in cell division, giving rise to the 45, X cells.

Nevoid hyper or hypopigmentation refers to a pattern of hypopigmentation or hyperpigmentation of the skin distributed along the lines of Blaschko. Among children with this finding, as many as a third will have other congenital anomalies. In this group, as many as 60% have been shown to be mosaic for chromosomal abnormalities, either in lymphocytes, in fibroblasts, or in both. This condition has been frequently referred to in the literature as “hypomelanosis of Ito,” but this designation implies a single disease entity or cause, whereas mosaicism for many different chromosomal abnormalities has been reported. Mosaicism may involve both normal and abnormal cell populations, such as 46, XY/47, XY, +18, or two abnormal cell populations, such as 45, X/46, X, +ring X. Mosaicism for triploidy (46, XY/69, XXY) can also cause hypopigmentation along the lines of Blaschko. Mosaicism for ostensibly balanced (i.e., all the chromosomal material is present, it is just rearranged in an abnormal fashion) chromosomal translocations, where one of each member of two different chromosome pairs have exchanged pieces, has also been shown to cause nevoid hypo/hyperpigmentation. Taibjee and colleagues (10) have suggested that the genome has many genes responsible for pigment production and that the chromosomal abnormalities demonstrated in hypomelanosis of Ito all involve regions where pigment genes are disrupted, but this does not explain the patterns seen in chimeras, who presumably have two normal, albeit distinct, genomes.

*Functional mosaicism* for genes on the X chromosome occurs in human females because early in embryogenesis, perhaps at about the 32 cell stage of development, one or the other of the two X chromosomes inactivates or turns off. This is true for most of the regions on the X, although a few areas, including the region with the gene for sterol sulfatase, mutations in which cause X-linked ichthyosis, escape inactivation.

This inactivation is random, the maternal and the paternal X have equal chances of being inactivated, and it is permanent. Thus, females who are carriers for X-linked recessive conditions may express the condition to varying degrees depending on the proportion and distribution of cells that are expressing the X chromosome with the mutation. In X-linked dominant disorders, affected females are spared the lethality seen in males because of the presence of some cells in which the X chromosome without the mutation is active and thus maintains normal function. Males with Klinefelter syndrome also undergo X-inactivation and thus they may survive in the presence of a lethal X-linked gene because some cells will have an active normal X chromosome.

Focal dermal hypoplasia (FDH: Goltz syndrome) is an X-linked dominant disorder characterized by asymmetric, atrophic, hyperpigmented, or hypopigmented, linear streaks and punctuate cribriform scarring and telangiectases along the lines of Blaschko. Fat herniations appear as linear arrays of yellow nodules. Osteopathia striata is the characteristic radiologic feature, and cutaneous or bony syndactyly is common. In addition, eye abnormalities and dental defects are typical. Focal dermal hypoplasia is generally lethal in hemizygous males. Females survive because of X inactivation, because not all cells in the body express the mutated gene. There have been a few males reported with FDH likely either caused by Klinefelter syndrome (47, XXY) or by a mutation during embryologic development, making the individual mosaic for the condition, thus escaping lethality (11).

Incontinentia pigmenti (IP) is another X-linked dominant condition with similar linear patterns of expression on the skin because of functional mosaicism. It is characterized in the neonatal period by blisters on an erythematous base along the lines of Blaschko (Fig. 1). This is followed by a verrucous phase, then a phase characterized by swirly hyperpigmentation, and ultimately by hypopigmentation and drop-out of follicles and sweat glands. Incontinentia pigmenti is caused by mutations in the *NEMO* gene (NF-kappa B essential modulator) located on the X chromosome (12). The mutation is usually lethal in utero in hemizygous males, resulting in spontaneous abortion. Occasionally, males survive, presumably by one of the same mechanisms – postzygotic mutations (mutations occurring after fertilization) and Klinefelter syndrome (47, XXY).

X-linked recessive hypohidrotic ectodermal dysplasia (HED/EDA1) is manifest in hemizygous males who have only one X chromosome, but are often not clinically apparent in carrier females. However, females can show subtle clinical features due to mosaicism caused by X inactivation, for example, having areas of affected skin in which the normal X is inactivated and only the X



**Figure 1.** Linear brown verrucous papules demonstrating the lines of Blaschko in a patient with incontinentia pigmenti. Photograph courtesy of Ilona J. Frieden, MD.

chromosome with the mutant gene is expressed. This has been shown in female carriers of X-linked recessive hypohidrotic ectodermal dysplasia (HED) (Christ-Siemens-Touraine syndrome), a severe disorder of the hair, sweat glands, and teeth. Using a starch iodine test, a mosaic distribution of functional sweat glands has been demonstrated along the lines of Blaschko in female carriers (13). Abnormalities of vellus hairs are seen in the same distribution. The authors suggest that testing to show the distribution of sweat glands in females with signs of hypohidrotic ectodermal dysplasia serves as a good tool to distinguish X-linked HED from autosomal recessive HED, because in the latter, homozygous females have a complete absence of sweat glands, and heterozygotes

for the autosomal recessive form do not have partial manifestation.

What is shared in common by all these forms of somatic mosaicism: single gene, chromosomal, or X-linked, is that the cutaneous manifestations of mosaicism most often follow the lines of Blaschko.

The lines of Blaschko were first described by the German dermatologist, Alfred Blaschko, in 1901. He recognized lines and whorls of pigmentary and nevoid genetic skin conditions in a V shape on the back that he called the "fountain spray" and an S-shape on the abdomen. This distribution differs from dermatomes, and it has been hypothesized that this pattern may depict the destined and directed route of embryonic ectodermal cell migration, namely keratinocytes and melanocytes (14,15). The extent of skin involvement may depend on the stage of development during which the mutation or chromosomal nondisjunction took place, or on the proportion and distribution of cells that have the normal X inactivated. If the mutation occurs early in embryologic development, then widespread skin patterning may result. If the event takes place later in development, then the mosaicism may be limited to a more confined anatomic region.

Pigmentary mosaicism can also present in several other patterns, including phylloid (meaning leaflike), checkerboard, and patchy pigmentation without midline separation (16).

## OTHER MECHANISMS OF MOSAICISM

### Revertant mosaicism

Recently, several examples of revertant mosaicism have been described (17–21). Revertant refers to the reversal or correction of a mutation; in other words, a restoration of the wild-type amino acid sequence. When this occurs either during embryogenesis or when the onset is after birth, patches or localized areas of skin show the revertant phenotype on a molecular and clinical level. This mechanism was first described in 1997 for autosomal recessive (AR) non-Herlitz junctional epidermolysis bullosa (EB) when Jonkman et al (19) described a patient who was a compound heterozygote for nonsense and frame-shift mutations in type XVII collagen gene, *COL17A1*. In this case, expression of normal-type XVII collagen occurred in revertant patches of skin that were phenotypically normal and did not blister. The authors hypothesized that the rescue of the phenotype resulted from gene conversion, in which one allele converts the mutated sequence of the other allele to the wild-type sequence (possibly by nonreciprocal exchange).

Another mechanism for reversion is by mRNA rescue of a frame-shift mutation by a downstream second-site mutation. In this situation, the original mutation remains, but the second mutation restores the normal reading frame and allows for effective translation of the protein. If the expressed protein is normal, complete reversion or correction occurs. If the protein now being expressed is aberrant, partially impaired function results and is referred to as a partial revertant. This was demonstrated in another patient with AR non-Herlitz junctional EB. Keratinocytes obtained for culture by laser capture microdissection contained a second insertion downstream from the deletion that restored the reading frame (17). This change was only seen at the molecular level; the phenotype in the patient was not corrected. According to Jonkman (18), several factors are key in determining the revertant phenotype; these include cell lineage, the percentage of cells reverted, and the timing of the reversion, whether during embryogenesis or later in life. Revertant mosaicism is sometimes referred to as "natural gene therapy" and may have implications for future strategies for gene therapy.

### Epigenetic mosaicism

Epigenetic mosaicism refers to environmental factors, such as retrotransposons, that cause alterations in the genetic material. Retrotransposons are viral DNA sequences that are incorporated into the nuclear DNA, are replicated, and regulate local genes often by silencing or activating a gene through methylation or demethylation (22). This has been shown to be important in coat color in mice and dogs, but has not been demonstrated in humans. Epigenetic events may play a role in the development of cancer.

### Chimeras

A chimera is an organism created when two zygotes (usually both normal) are fused, resulting in an individual who is composed of two genetically distinct cell populations. Although, technically, chimeras are not mosaic because the presence of distinct cell populations is not the result of events arising in a single zygote, we include it here because it can also manifest with segmental pigment patterns on the skin.

### CONSIDERATIONS ON SKIN BIOPSIES

Skin biopsy to obtain cell lines to test for mosaicism can be challenging. One must consider which cell lines are likely to be affected when establishing the cell culture and which molecular study will have the greatest

sensitivity to detect the mutation. Gonosomal mutations can usually be detected from peripheral blood lymphocytes, whereas the blood is unlikely to show a mutation in somatic mosaicism that has occurred later in embryogenesis. Disorders that follow the lines of Blaschko are expressing in ectodermal derivatives and therefore keratinocyte or melanocyte culture will likely have the highest yield (15). In segmental neurofibromatosis, in which neurofibromas predominate in a dermatomal pattern, fibroblast culture is likely to reveal the mutation.

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## Neurofibromatosis 1

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*Karen Stephens, PhD*

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### INTRODUCTION

Among genomic disorders, submicroscopic deletions underlying neurofibromatosis 1 (NF1) are unusual because they involve the deletion of a tumor suppressor gene (*NF1*), they show a different preference for low-copy repeats (LCR) as substrates for meiotic vs mitotic recombination events, and they account for only a small fraction of mutations that cause the disorder. The *NF1* gene at chromosome 17q11.2 is flanked by two sets of LCRs in direct orientation that undergo paralogous recombination. A pair of NF1-REPs mediate the recurrent constitutional 1.4-Mb microdeletion that occurs preferentially during maternal meiosis, whereas a pair of *JJAZ1* pseudogene and functional gene mediate the recurrent 1.2-Mb microdeletion that occurs preferentially during postzygotic mitosis in females. Breakpoints have been mapped at the nucleotide level for both deletions and sequence features that may contribute to the choice of discrete sites for strand exchange have been identified. NF1-REP-mediated *NF1* microdeletions involve 13 additional genes, whereas *JJAZ1*-mediated microdeletions involve the same genes but one. *NF1* microdeletions are of great interest because they predispose to a heavy tumor burden, malignancy, and possibly other severe manifestations.

In 1992, the first report of a NF1 patient with a submicroscopic contiguous gene deletion spanning the *NF1* tumor suppressor gene provided direct evidence that this common autosomal dominant disorder was caused by haploinsufficiency of the *NF1* protein product, neurofibromin (1). Here, subsequent molecular, genetic, and clinical studies of *NF1* microdeletions are reviewed, which have made significant contributions to our understanding of the mutational mechanisms and pathogenesis of this common multisystemic, progressive, tumor predisposition disorder, and to genomic disorders in general.

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## MOLECULAR BASIS OF NEUROFIBROMATOSIS 1

Virtually all subjects affected with NF1 develop multiple benign neurofibromas, which are tumors of superficial and deep peripheral nerves that increase in number with age, along with pigmentation changes of café au lait macules, axillary/inguinal freckling, and hamartomas of the iris of the eye (2). Neurofibromas are unpredictable with regards to number, location, rate of growth, and potential for malignant transformation. Additional complications unrelated to neurofibroma development are legion, and include learning disabilities, scoliosis and other bone abnormalities, optic glioma, and malignancies of various organ systems, such as malignant peripheral nerve sheath tumors (MPNST), rhabdomyosarcoma, and myeloid leukemias (3). Although a deletion or other constitutional inactivating mutation of one *NF1* allele predisposes to benign or malignant tumorigenesis, a somatic inactivating mutation of the remaining *NF1* allele in a tumor progenitor cell is an early, if not initiating, event in most, if not all, NF1-associated tumors. The tumor progenitor cell of the cellularly heterogeneous neurofibroma and the MPNST is the Schwann cell (4). A second requirement for neurofibroma development in a mouse model is the presence of heterozygous *Nf1*<sup>+/-</sup> murine cells in the micro-tumor environment (4). The requirement of different neurofibromin levels in target cells vs supportive cells results in modulation of the RAS signaling pathway, as neurofibromin is a guanosine-5'-triphosphatase (GTPase)-activating protein that catalyzes the conversion of active RAS-GTP to inactive RAS-GDP (5). The requirement for *Nf1* heterozygosity in the mouse brain for the development of optic nerve glioma by *Nf1* ablated astrocytes (6) extends the emerging paradigm that neurofibromin haploinsufficiency in cells of the micro-tumor environment is critical.

### *The NF1 Mutational Profile*

The mutational profile of the *NF1* gene at chromosome 17q11.2 is complex, as both constitutional and somatic mutations occur that result in generalized or segmental (localized) NF1 disease and tumor development (Table 1). NF1 is inherited as an autosomal dominant trait, but also occurs sporadically in approx 30–50% of cases. In 80–90% of cases owing to intragenic *NF1* inactivating mutations, no correlation has been detected between mutation type and/or location and the development of specific manifestations (7–9). Approximately 5% of NF1 cases are because of submicroscopic, contiguous gene deletions (10–12). Most *NF1* microdeletions are *de novo* and are predominantly of maternal origin, although familial cases do occur (13–17). Subjects with *NF1* microdeletions tend to have a high tumor burden and may have facial anomalies, early age at onset of dermal neurofibromas, vascular anomalies, learning disabilities, astrocytomas, and malignancy (12–14,18,19). Formal studies regarding *NF1* microdeletion genotype/phenotype correlations await a comprehensive clinical and molecular evaluation of a cohort of *NF1* microdeletion subjects ascertained in an unbiased manner. One such study has shown that *NF1* microdeletion doubles the lifetime risk of MPNST relative to nondeletional genotypes (20). One confounding factor in such studies is that approx 25% of *NF1* microdeletions occur as postzygotic mutations and different levels of mosaicism in patient tissues can result in generalized or localized NF1 (*see* LCR-Mediated Somatic *NF1* Microdeletions) (21). A major area of research is focused on identifying genes in the deleted region that modify NF1-related tumorigenesis or other manifestations of the disorder. As summarized in Table 1, somatic second hit inactivating mutations of *NF1* are typically owing to loss of heterozygosity by recombination, chromosomal deletion that is not known to be LCR-mediated or intragenic inactivating mutations (Table 1).

Table 1  
Mutational Profile of the *NF1* Gene

NF1 <i>mutation</i> <i>category</i>	<i>Patient</i> <i>phenotype/</i> <i>tissue</i>	<i>Mode of</i> <i>inheritance</i>	NF1 <i>mutation type</i>	<i>Selected</i> <i>references</i>
Constitutional	NF1	familial or <i>de novo</i>	LCR-mediated contiguous gene deletion (approx 5%) Nonsense, splicing defects, missense (85–90%)	10 39–41
Somatic mosaicism	NF1	<i>de novo</i>	LCR-mediated contiguous gene deletion	21,38,42–44
	Localized NF1	<i>de novo</i>	LCR-mediated contiguous gene deletion, multi-exonic deletion	21,38,45
Germline mosaicism	Unaffected	<i>de novo</i>	Intragenic deletion	46
Somatic 2nd hits in tumor tissue <sup>a</sup>	Neurofibroma	<i>de novo</i>	Splicing defects, LOH <sup>b</sup>	47–49
	MPNST	<i>de novo</i>	LOH <sup>b</sup> , deletion	48–52
	Myeloid leukemia	<i>de novo</i>	Nonsense, others that predict premature truncation	7

<sup>a</sup>Reviewed in ref. 52.

<sup>b</sup>Loss of heterozygosity via recombination or deletion.

### RECURRENT NF-REP-MEDIATED *NF1* MICRODELETIONS

An estimated (50–60%) of constitutional *NF1* microdeletions have a common recurrent 1.4-Mb deletion that includes *NF1* and at least 13 other genes (Fig. 1; Table 2) (11). Both centromeric and telomeric deletion breakpoints cluster within two LCR sequences, or paralogs, termed NF1-REP-P1 and NF1-REP-M (Fig. 1). The direct orientation of these two NF1-REPs suggested a mechanism consistent with the existing paradigm whereby deletions occur by nonallelic homologous recombination, or paralogous recombination, between misaligned paralogs on the interchromosomal, intrachromosomal, or intrachromatid level (22). For *NF1*, this mechanism was confirmed by mapping NF1-REP-mediated deletion breakpoints at the nucleotide level (17,23) and by haplotype analyses (16).

NF1-REPs are complex assemblies of paralogs from different sequence families consisting of expressed genes, pseudogenes, gene fragments, and non-coding sequence (Fig. 2) (24). NF1-REP-P1 and NF1-REP-M share a 51-kb segment of 97.5% sequence identity, termed NF1-REP-51, which serves as the substrate for paralogous recombination events. Two other components of the NF1-REP family include NF1-REP-P2, which is centromeric to *NF1*, and NF1-REP-E19 located on chromosome 19p13.13 (Fig. 2) (24,25). *NF1* microdeletions owing to apparent paralogous recombination between NF1-REP-P2 and -M have been reported, but their breakpoints have not been mapped at the nucleotide level (26). Large kilobase-sized polymorphisms have not been observed in the NF1-REPs, although certain structural features, such as the inverted repeats in *KIAA0563rel-ψ* of NF1-REP-P1 that could mediate an inversion event, may generate such polymorphisms in the general population. There is no evidence of constitutional or somatic chromosomal translocations that could be attributable to recombina-

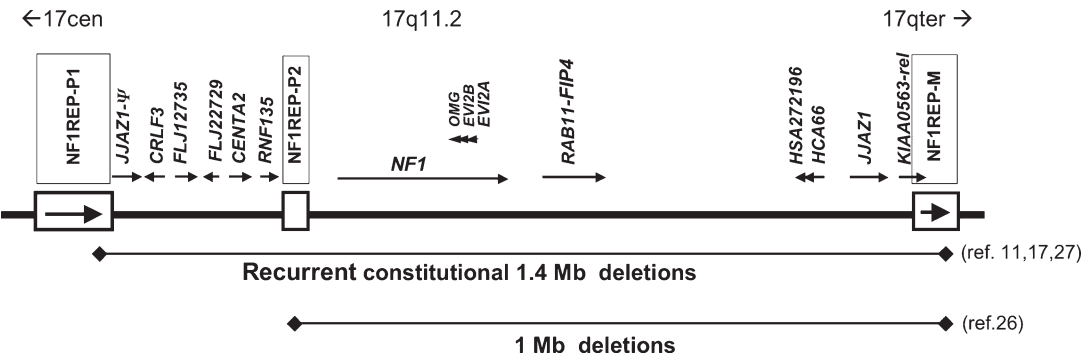


Fig. 1. Genomic structure of the *NF1* microdeletion region in chromosome 17q11.2 and the recurrent constitutional 1.4-Mb deletion. The 350-kb *NF1* gene, and other regional genes, are indicated by arrows showing their direction of transcription. *OMG*, *EVI2A*, and *EVI2B* are within an *NF1* intron and are transcribed on the alternate strand. Table 2 details the genes in this region. Boxes designate the 3 NF1-REPs, with arrows indicating direct repeat orientation of NF1-REP-P1 and -M. Genomic length from NF1-REP-P1 through NF1-REP-M is 1.5 Mb and the most frequent constitutional deletions are 1.4 Mb. Two approx 1-Mb deletions have been reported apparently because of recombination between NF1-REP-P2 and -M (see Recurrent NF-REP-Mediated *NF1* Microdeletions; 26). NF1-REP-P1, -P2, -M are 131, 43, and 75 kb in length, respectively (see Fig. 2 for details). Gene names are from May 2004 assembly of the human genome (<http://genome.ucsc.edu/>) and may differ from names on previously published maps of the region (23,25,26).

Table 2  
Functional Genes Within the Recurrent 1.4-Mb *NF1* Microdeletion Region

Gene/marker <sup>a</sup>	Description	REFSEQ mRNA accession number
<i>CRLF3</i>	Cytokine receptor-like factor 3	NM_015986.2
<i>FLJ12735</i>	Putative ATP(GTP)-binding protein <sup>b</sup>	NM_024857.3
<i>FLJ2279</i>	Hypothetical protein, unknown function	NM_024683.1
<i>CENTA2</i>	Centaurin alpha 2, binds phosphatidylinositol 3,4,5-triphosphate and inositol 1,3,4,5,-tetrakisphosphate	NM_018404.1
<i>RNF135</i>	Ring finger protein 135	NM_032322.3
<i>NF1</i>	Ras GTPase stimulating protein	NM_197939.1
<i>OMG</i>	Oligodendrocyte myelin glycoprotein; cell adhesion molecule contributing to myelination in the central nervous system	NM_000267.1
<i>EVI2B</i>	Ecotropic viral integration site 2B, membrane protein	NM_002544.2
<i>EVI2A</i>	Ecotropic viral integration site 2A, membrane protein	NM_006495.2
<i>RAB11-FIP4</i>	Putative Rab11 family interacting protein 3	NM_014210.1
<i>HSA272196</i>	Hypothetical protein, unknown function	NM_032932.2
<i>HCA66</i>	Hepatocellular carcinoma-associated antigen 66	NM_018405.2
<i>JJAZ1</i>	Hepatocellular carcinoma-associated antigen 66	NM_018428.2
	Protein with zinc finger domain and homology to Drosophia Polycomb protein SUZ12	
<i>KIAA0563-rel</i>	KIAA0563-related, unknown function	NM_015355.1
		NM_014834.2

<sup>a</sup>From the May 2004 genome assembly (<http://genome.ucsc.edu/>).  
<sup>b</sup>ATP, adenosine-5'-triphosphate; GTP, guanosine-5'-triphosphate.

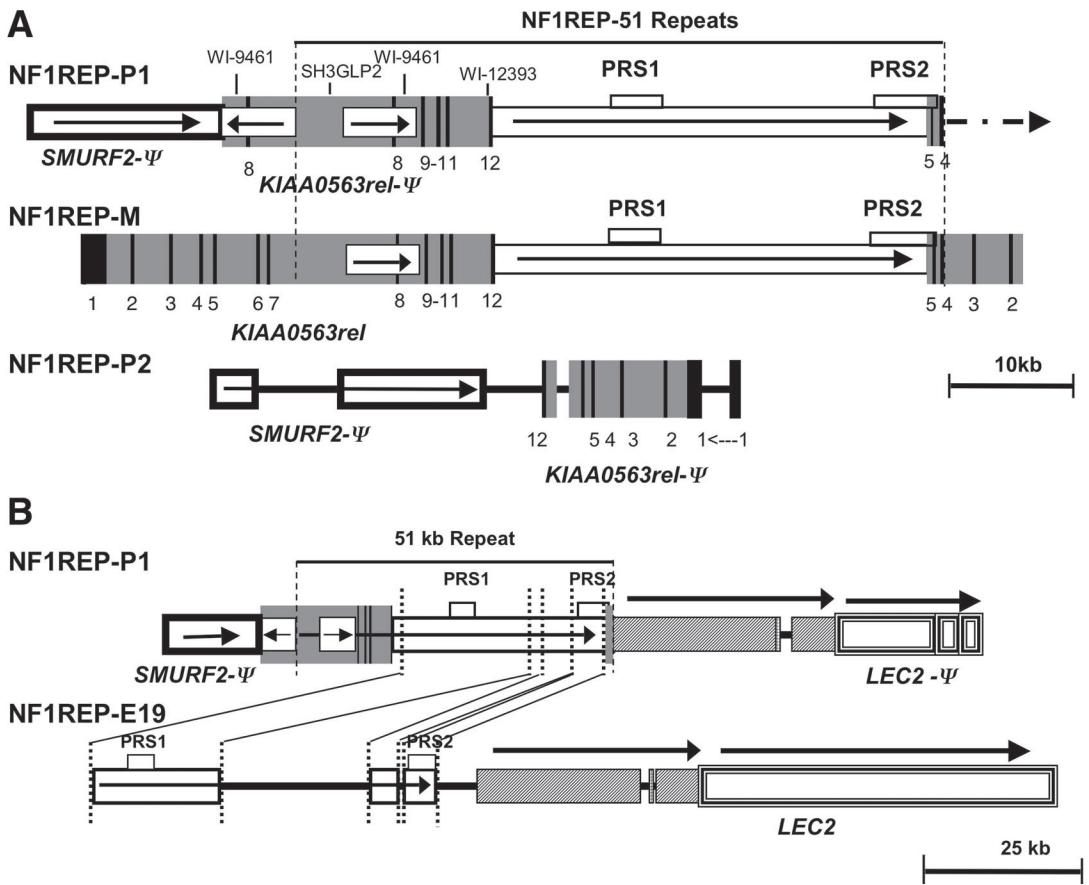


Fig. 2. The structure of the NF1-REP paralogs. (A) The structure of NF1-REP-P1 (131 kb) and a partial structure of NF1-REP-M (see B for complete structure) are shown. These serve as paralogous recombination substrates for the common 1.4-Mb *NF1* microdeletion. The 51-kb high sequence identity region, designated NF1-REP-51 harbors the recombination hotspots PRS1 and PRS2. Gray blocks indicate the *KIAA0563rel* functional gene and related pseudogene ( $\psi$ ) fragments with numbered black bars designating exons or exon-derived sequences. White boxes with arrows inside *KIAA0563rel* sequences designate the orientation of a 5.9-kb inverted repeats with two copies in NF1-REP-P1 and one in NF1-REP-M. STS in *KIAA0563rel* are shown as landmarks. Open blocks with bold margins are *SMURF2*-derived pseudogene ( $\psi$ ) fragments. The arrow at the telomeric end of NF1-REP-P1 indicates that it is truncated; see panel B for full-length structure. NF1-REP-P2 is a partial NF1-REP with fragments derived from *SMURF2* and *KIAA0563* pseudogenes. Figures are oriented from centromere (left) to telomere. BAC identities and accession numbers for each NF1-REP are given in Forbes et al. (24). (B) Comparison of NF1-REP-P1 and NF1-REP-E19, at chromosome 19p13.13. Boxes are labeled as in (A), in addition to *LEC2* and its pseudogene (open blocks with borders of multiple lines) and non-coding sequences between PRS2 and *LEC2* (blocks filled with diagonal lines); sequence orientations are shown with arrows. The *SMURF2*, non-coding, and *LEC2* sequences flanking the 51-kb repeat are considered part of NF1-REP-P1 based on paralogy with NF1-REP-P2 and NF1-REP-E19. BAC identities and accession numbers for each NF1-REP are given in Forbes et al. (24). Note difference in scales between panels A and B. (Adapted with permission from ref. 24.)

tion between chromosome 17 NF1-REPs and NF1-REP-E19, although NF1-REP-P1-51 and NF1-REP-E19 share 94–95% sequence identity that includes the recombination hotspots PRS1 and PRS2 (*see* next section) (Fig. 2) (24). Additional NF1-REP-like elements with KIAA0563 fragments are at chromosome 17q12 and 17q24, but they do not share any other sequences with NF1-REP-P1, -P2, or -M (11,24,25). Whether these LCRs mediate chromosomal rearrangements is unknown. Similar to those of other LCRs that mediate genomic disorders on chromosome 17, the segmental duplications giving rise to NF1-REPs originated in recent hominoid evolution about 25 million years ago before the separation of orangutan from the human lineage (25). Fluorescent *in situ* hybridization (FISH) studies showed the presence of NF1-REP-P1 and NF1-REP-M orthologs flanking the *NF1* gene in the Great Apes (27).

### ***Discrete Paralogous Recombination Sites***

Despite 51 kb of 97.5% sequence identity between NF1-REP-P1-51 and NF1-REP-M-51, paralogous recombination occurred preferentially at two discrete sites. *NF1* microdeletion breakpoints were mapped at the nucleotide level to intervals defined by paralogous sequence variants (PSV; also known as NF1-REP-specific variants). The product of paralogous recombination is a chimeric NF1-REP-P1/NF1-REP-M and shows a pattern of PSVs with transition from NF1-REP-P1 PSVs to NF1-REP-M PSVs at the breakpoint interval. Breakpoint mapping was facilitated by use of human/rodent somatic hybrid cell lines that carried only the deleted homolog 17 of the patient (17,23). Sixty-nine percent ( $N = 78$ ) of *NF1* microdeletion cases had breakpoints that clustered at paralogous recombination sites 1 and 2 (PRS1 and PRS2) (Fig. 3) (17,23,24) (23). PRS2 harbored 51% of breakpoints, whereas PRS1 harbored 18%. A single case UWA160-1 had a distinct breakpoint centromeric to PRS sites (Fig. 3). The PRS1 and PRS2 regions are 4.1 and 6.3 kb in length, respectively, and are 14.5 kb apart. Each PRS has a hotspot where the majority of breakpoints mapped; PRS2 has a 2.3-kb hotspot that accounts for 93% of breakpoints, whereas PRS1 has a 0.5-kb hotspot accounting for 60% of breakpoints. During sequence analysis of recombinant PRS in several cases, instead of a perfect transition of PSVs from NF1-REP-P1 to -M, the PSVs were in “patches” with a complex transition from NF1-REP-P1 to -M to -P1 to -M (17,23), indicating apparent gene conversion events. These regions were relatively short (<627 bp) and, like similar events of REP-mediated rearrangements in CMT1A and *AZF*a and *IDS* (28–31), are considered consistent with a mechanism of double-strand break repair.

There was no significant difference between PRS1- and PRS2-mediated microdeletions for the parent of origin or for *de novo* vs familial cases (17,23). In a series of 59 *NF1* microdeletion cases for which clinical evaluation of the parents was available, 10% inherited the disease, and presumably the microdeletion, from an affected parent. Among 45 *de novo* cases where parental origin could be determined, 80% were of maternal origin. The recent development of deletion-specific amplification assays that detect the recurrent *NF1* microdeletions at PRS1 and PRS2 will facilitate the assembly of patient cohorts of the same genotype for clinical evaluation and will quickly identify those patients with variant deletions, which will be important to narrow the critical region of the deletion responsible for the increased tumor load and malignancy risk of microdeletion patients (17,23).

### ***Genomic Context and Sequence Analysis of Paralogous Recombination Sites***

Detailed analysis of the PRS and the NF1-REP-51 paralogs at the nucleotide level identified interesting features, but lacked compelling evidence for why breakpoints preferentially occur at these sites (24). There were no obligate local sequence features shared by PRS1 and PRS2.

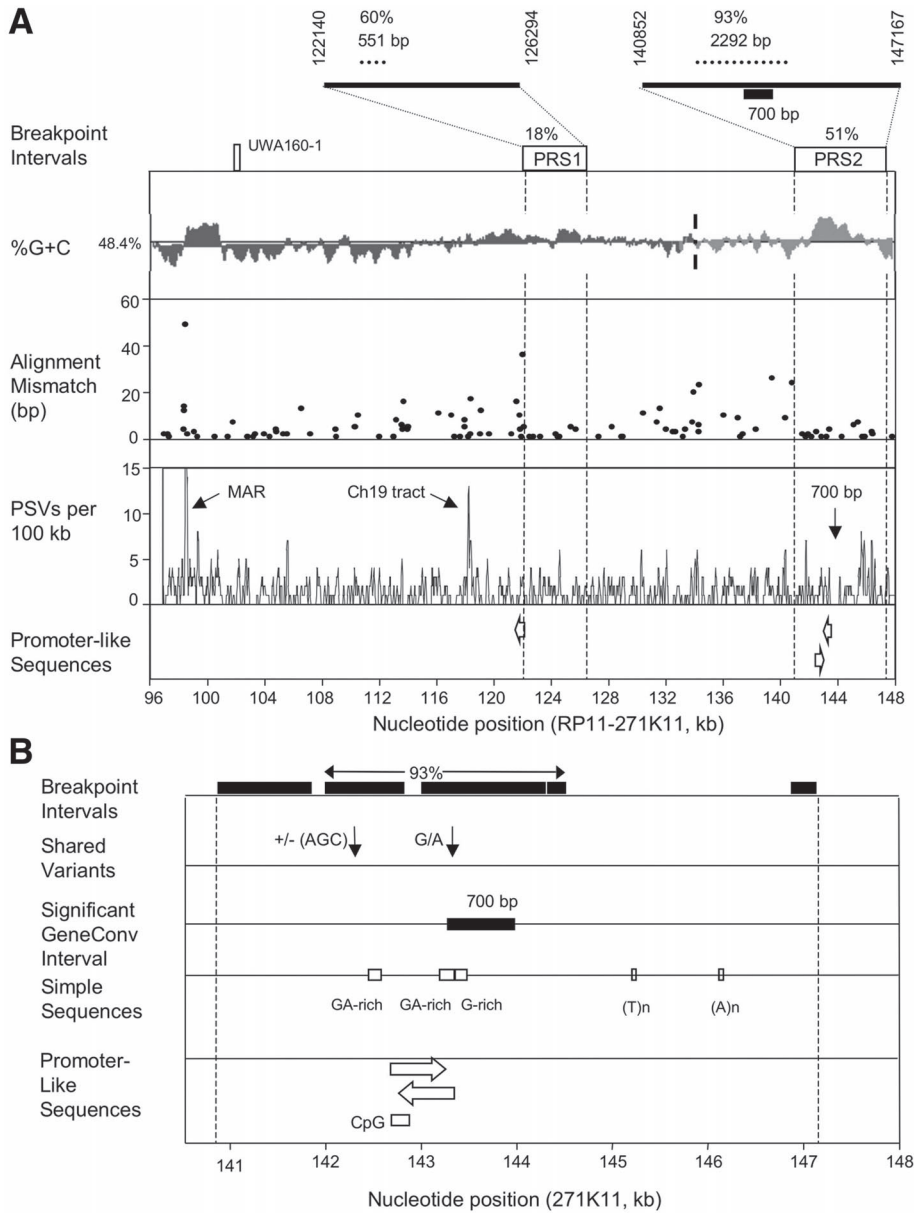


Fig. 3. Genomic context of the paralogous recombination hotspots for *NF1* microdeletion. (A) Alignment, identity, and sequence features of the 51-kb high identity *NF1*-REP-P1-51 and *NF1*-REP-M-51 paralogs. Alignment mismatch panel shows gap alignments ranging from 1 to 50 bp between *NF1*-REP-P1-51 and *NF1*-REP-M-51 in BACs RP11-271K11 and RP11-640N20, respectively. PSVs for each REP, indels excluded, with a sliding 100-bp window are shown, including a matrix attachment site (MAR), an apparent gene conversion tract with variants matching *NF1*-REP-E19, and a 700-bp segment of perfect match with statistical evidence of gene conversion. The positions of promoter like sequences not associated with known genes are indicated in the lower panel. (B) Detailed structure of the PRS2 region. Breakpoint intervals are shown along with the 2.3-kb hotspot, which harbors 93% of breakpoint intervals in PRS2 region. Finer localization of the 700-bp gene conversion tract and the promoter like sequences from (A) are shown. Nucleotide positions for both panels refer to the *NF1*-REP-P1-51 in BAC RP11-271K11. (Adapted from ref. 24 with permission.)

Basic Local Alignment Search Tool (BLAST; [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)) comparison of PRS1 and PRS2 showed no significant sequence identity with the exception of *Alu* elements, LINES and other high-copy repeats, which typically shared less than 80% identity in short segments (24). PRS1 and 2 regions have quite different patterns of G+C content; the PRS2 hotspot is very G+C rich, while the PRS1 hotspot is not (Fig. 3). Both PRS hotspots are 1–2 kb distal to relative large alignment gaps (Fig. 3A), yet these did not suppress pairing as recombination in at least a few cases occurred within less than 1 kb from the gaps. The PRS regions are not of greater or lesser paralogous sequence identity as shown by the spatial distribution of PSVs, which are relatively evenly distributed across the NF1-REP-51 segment (Fig. 3) (24). Numerous tests for the presence of motifs with demonstrated or suspected roles in recombination, transcription, or translation were performed. A *Chi* element within PRS2 was identified (17), but this association is not preferential, as it is one of four evenly spaced *Chi* elements in NF1-REP-51 (24). Figure 3B shows the location of a CpG island and two promoter-like sequences, which although not associated with any known gene, may function to provide chromatin accessibility. Statistical tests for gene conversion identified the 700-bp perfect match between NF1-REP-P1-51 and NF1-REP-M-51 that coincided with PRS2 (Fig. 3B). Although perfect sequence match may contribute to breakpoint localization, these results suggest that perfect tracts at paralogous recombination hotspots may be a result of gene conversion at sites at which preferential pairing occurs for other unknown reasons (24). A search for palindromes, which are associated with other genomic rearrangements (32–35), found no palindromes larger than 18 bp and separated by 63 bp. The palindromes within the KIAA0563- $\psi$  of NF1-REP-P1 are considered too distant to influence recombination at the PRS regions (Fig. 2A).

### UNIQUE, NON-LCR-MEDIATED *NF1* MICRODELETIONS

A subset of submicroscopic *NF1* microdeletions have breakpoints outside of the NF1-REP paralogs (Fig. 4) and appear to arise by a mechanism other than paralogous recombination. Among the five larger deletions, all except the BUD case (patient B in Fig. 4) have a telomeric breakpoint within the *ACCN1* gene. BUD has a telomeric breakpoint in the *SLFN* gene cluster. At least four of these deletions also have different centromeric breakpoint intervals. Only breakpoints in case six have been mapped at the nucleotide level with the centromeric breakpoint between *BLMH* and *CPD* and the telomeric breakpoint in *ACCN1* intron 1. The breakpoints were not located within LCR elements. However, there were stretches of 20–21 bp of *Alu*-like elements at the two breakpoints and LINE and short interspersed nuclear element (SINE) elements within several hundred bp from the breakpoints. Together, these data provide support for a mechanism of nonhomologous end-joining (NHEJ) (36). Because LCRs are not known to be located in/near the breakpoints of the other *NF1* microdeletions shown in Fig. 4, they may well arise by NHEJ also. As more such deletions are identified, it will be of interest to determine if there is a preferential parent of origin effect. In these examples, UWA106-3, BUD, and 96-2 have paternally derived deletions, whereas 372A was maternally derived. In general, larger deletions tend to occur in NF1 patients with severe or additional complications, but phenotypic information is limited and the extent of an *NF1* deletion has no predictive value to date.

The smaller deletions depicted on Fig. 4 are of interest because precise breakpoint mapping and full clinical evaluation of such patients may serve to narrow the critical region between NF1-REP-P1 and NF1-REP-M that confers the phenotype of heavy tumor load and increased

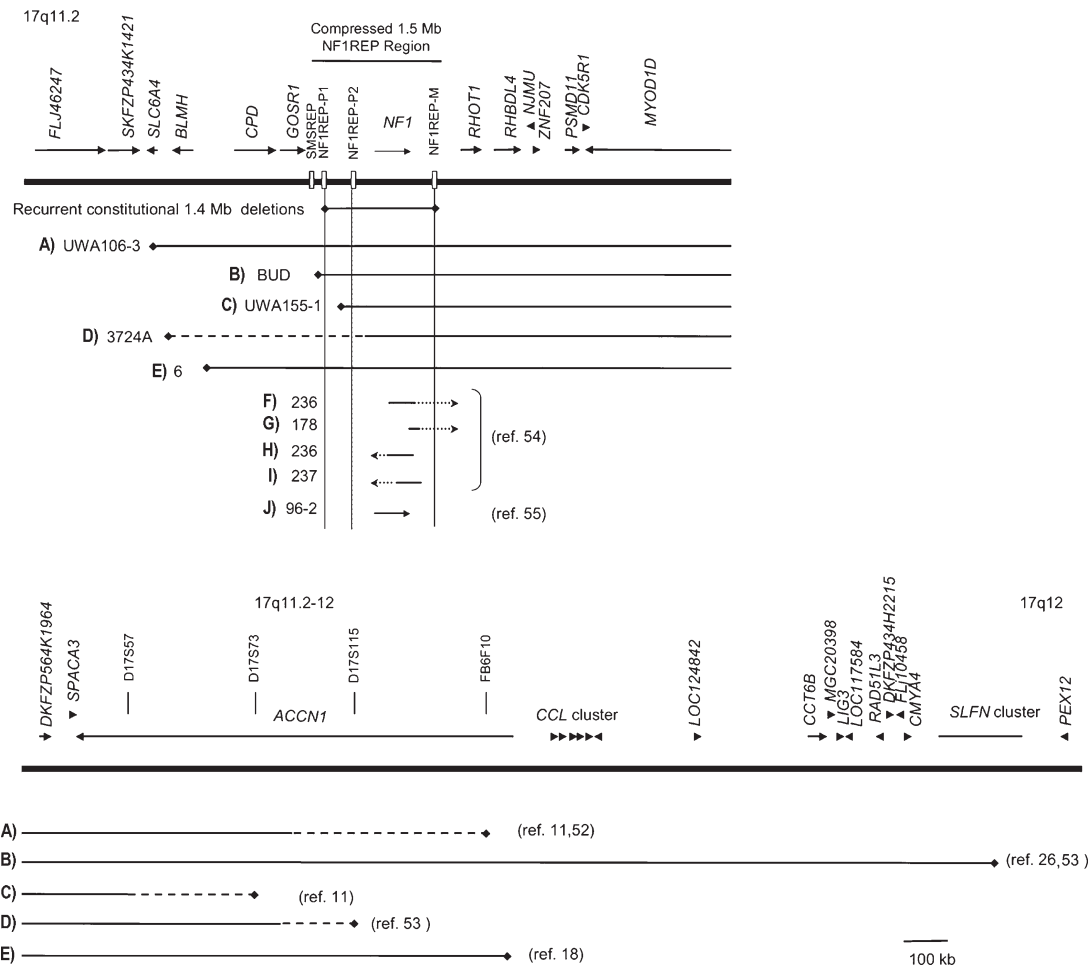


Fig. 4. Nonrecurrent *NF1* microdeletions. A schematic of the 5.6-Mb region from *FLJ46247* to *PEX12* is diagrammed in two panels. Figures are drawn to scale with the exception of the 1.5-Mb *NF1*-REP region, which is compressed for space considerations. Gene names and gene clusters are written above arrows indicating their direction of transcription. The three *NF1*-REP (open boxes) and an adjacent *SMS* REP (gray box) are indicated. Four markers within *ACCN1* are shown that serve to differentiate the extent of deletions with breakpoints within this large gene. Below the map, the recurrent 1.4-Mb deletion is shown compared to that of 10 cases (A–J) with unique breakpoints. Solid lines indicated deleted region and dashed lines indicate uncertainty in the precise endpoint (*see* text for details). Gene names in this figure are from a May, 2004 assembly of the human genome (<http://genome.ucsc.edu/>) and may differ from names on previously published maps of the region(23,25,26,36).

malignancy risk to deletion patients. Patients 236, 178, 236, and 237 have at least one breakpoint within the *NF1* gene, but the extent of the deletion and position of the other breakpoints are not known. Patient 96-2 is deleted for much of the *NF1* gene, but whether other genes are involved in this deletion is not known.

### LCR-MEDIATED SOMATIC *NF1* MICRODELETIONS

An estimated 25% of *NF1* microdeletions occur as postzygotic mutations during mitosis resulting in tissue mosaicism (21) and a phenotype that can vary from the classical generalized

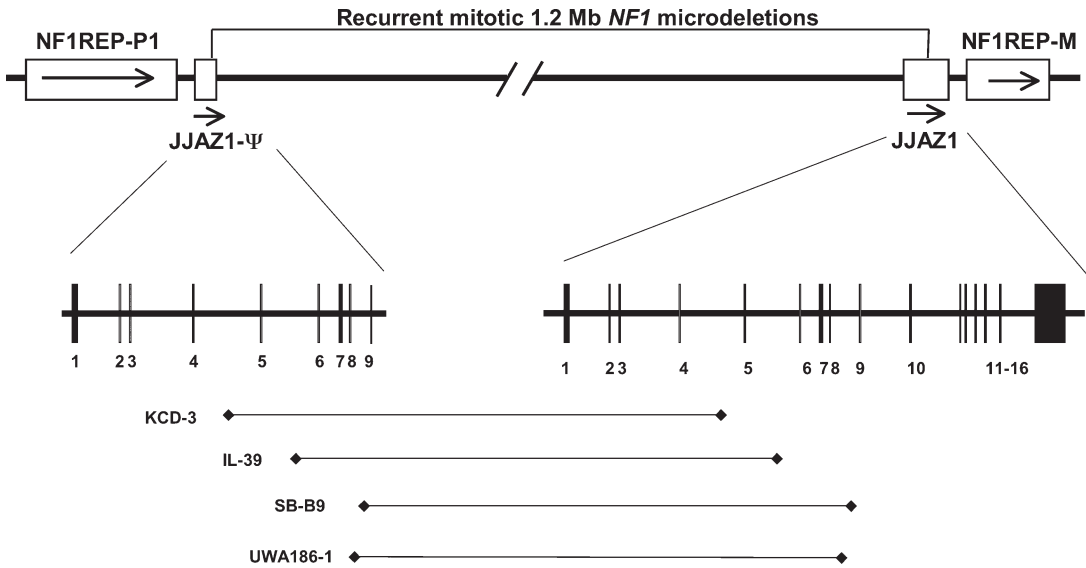


Fig. 5. Recurrent mitotic *NF1* microdeletions. The schematic shows the pair of *JJAZ1* low-copy repeats (LCRs) that mediate recurrent mitotic microdeletions of the *NF1* region (refer to Fig. 1 for genomic context of the region). These LCRs share 46 kb of homology with 97% sequence identity and are located just “internal” to the *NF1*-REP-P1 and -M(26). Breakpoints are drawn for four cases of 1.2-Mb deletions mediated by paralogous recombination between the *JJAZ1*- $\psi$  pseudogene and the *JJAZ1* functional gene. (Three from ref. 21 and UWA186-1 as an additional unpublished case from my laboratory.)

*NF1* to localized or segmental *NF1* (37,38). Like the common recurrent meiotic *NF1* microdeletions, somatic rearrangements occur by paralogous recombination; however, the site of preferential exchange was different. Seven of eight mitotic *NF1* microdeletions had breakpoints that clustered at the *JJAZ1*- $\psi$  pseudogene and the *JJAZ1* functional gene, which are direct repeats located adjacent and *NF1*-REP-P1 and *NF1*-REP-M (Fig. 1) (21). The *JJAZ1*- $\psi$  has 9 exons that share 46-kb homology at 97% identity with the functional *JJAZ1* gene (Fig. 5) (26). In three cases, breakpoint intervals were mapped at the nucleotide level by use of PSVs and the microdeletions occurred at different sites (Fig. 5). Consistent with this observation is the breakpoint of a somatic mosaic female patient in my laboratory, UWA186-1, whose breakpoint in intron 8 is approx 2 kb proximal to that of SB-B9 (Stephens, unpublished observations). The *JJAZ1*- $\psi$ /*JJAZ1* fusion product of the recombination is expressed in human-rodent somatic cell lines, but unlikely to be translated owing to stop codons in the pseudogene (21).

The level of somatic mosaicism for *JJAZ1*-mediated *NF1* microdeletions varied significantly in different patient tissues. The percentage of deleted cells as determined by FISH was quite high in peripheral blood (91–100%) and significantly lower in buccal cells or skin fibroblasts (51–59%) (21). These data suggest that a selective growth advantage of hematopoietic stem cells carrying *NF1* microdeletions. Different levels of mosaicism significantly compound both the diagnosis and counseling of patients with *JJAZ1*-mediated mosaic *NF1* microdeletions.

*JJAZ1*- and *NF1*-REP-mediated *NF1* microdeletions have striking differences and parallels. First, paralogous recombination at *JJAZ1* LCRs is preferentially mitotic, whereas that at *NF1*-REP LCRs is meiotic. Second, *JJAZ1* paralogous recombination is intrachromosomal in

two cases examined (21), whereas NF1-REP paralogous recombination is primarily interchromosomal (16). Third, small inverted repeats of 75–127 bp flank the intronic *JJAZ1* breakpoints in two cases and may cause double strand breaks by forming hairpins (21). Parallels between the two types of microdeletions include paralogous recombination, and deletion of the same set of contiguous genes, except for the functional *KIAA0563-rel* gene near NF1-REP-M, which is not deleted in *JJAZ1*-mediated rearrangements (Fig. 1). Furthermore, both paralogous recombination events occur preferentially in females for reasons that are not known (15,17,21).

## FUTURE DIRECTIONS

It will be important to determine the molecular basis for the different preferences for LCR substrates during meiotic vs mitotic recombination. Furthermore, does *JJAZ1*-mediated recombination contribute to somatic *NF1* second hit mutations at NF1-associated tumors? Does this site represent a mitotic recombination hotspot in the genome, perhaps in the female genome? And why is maternal recombination more prevalent for both LCR-mediated microdeletions? Clinical studies to identify the putative modifying gene that confers the increased risk for tumorigenesis and malignancy remain a priority. These studies will be facilitated by new assays and approaches to identify patient cohorts of the same deletion genotype and exclude mosaic cases that would confound the analyses.

## SUMMARY

Submicroscopic deletions at chromosome 17q11.2 underlying the common genetic disorder NF1 are of great interest because they predispose to a heavy neurofibroma burden, malignancy, and possibly other severe manifestations. The NF1 microdeletion phenotype, which remains to be defined in detail, is thought to be owing to the deletion of the *NF1* tumor suppressor gene and an additional unidentified flanking gene(s). Surprisingly, there is a different preference for LCR recombination substrates for recurrent meiotic versus recurrent mitotic *NF1* microdeletion events. Paralogous recombination between a pair of 51-kb NF1-REPs mediate the recurrent common constitutional 1.4-Mb microdeletion that occurs preferentially during maternal meiosis. Recombination between the *JJAZ1* pseudogene and functional gene mediate the recurrent 1.2-Mb microdeletion, which occurs preferentially during postzygotic mitosis in females. NF1-REP-mediated *NF1* microdeletions involve 13 additional genes, whereas *JJAZ1*-mediated microdeletions involve the same genes but one. Breakpoints of both deletions mapped at the nucleotide level identify several potential sequence features that may contribute to the choice of discrete sites for strand exchange.

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## Chapter 17

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# Clinical molecular genetics of the neurofibromatoses

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### Summary

The neurofibromatoses are a heterogeneous group of genetic disorders that share a predisposition to the development of tumours of the nerve sheath. There are three major types – neurofibromatosis type 1 (NF1), neurofibromatosis type 2 (NF2), and schwannomatosis. Each of these disorders is genetically distinct and is caused by defects in different genes. This chapter focuses on the clinical value of, and issues related to, DNA based testing for NF1 and NF2. In the past 5 years, significant advances have been made in understanding the types of mutation that inactivate the *NF1* and *NF2* genes and their clinical consequences. This has led to the development and availability of sensitive and specific DNA-based tests. These tests have already altered the clinical diagnosis, management, and outcome for at least a subset of affected patients.

### Introduction

The neurofibromatoses are a heterogeneous group of genetic disorders that share a predisposition to the development of tumours of the nerve sheath. There are three major types, known as neurofibromatosis type 1 (NF1), neurofibromatosis type 2 (NF2), and schwannomatosis. Each of these disorders is genetically distinct and caused by defects in different genes (Table 1). In this chapter, I will focus on the clinical value of, and issues related to, DNA-based testing for NF1 and NF2. Currently, there is no molecular test available for schwannomatosis, as the gene is yet to be identified. An excellent review of this form of neurofibromatosis has recently been published (MacCollin *et al.*, 2005).

### Molecular basis of NF1 and NF2

NF1 is a progressive disorder with an unpredictable course, even among family members carrying the same mutation. The common features of NF1 are café au lait macules, intertriginous freckling, Lisch nodules, and multiple neurofibromas, although learning disabilities, bony abnormalities, and numerous other features and complications are not uncommon (reviewed in Friedman *et al.*, 1999; Friedman, 2002; Friedman, 2004). The clinical criteria for a diagnosis

of NF1 are well established and widely used (Debella *et al.*, 2000). Patients can develop several different forms of neurofibroma (Woodruff, 1999), which are benign tumours of the peripheral nerve sheath. Virtually all patients develop cutaneous neurofibromas, which typically appear in the second decade of life, grow slowly, increase in number with age, and are considered to carry a low risk of transformation into malignant peripheral nerve sheath tumours (MPNST; formerly known as neurofibrosarcomas). The types of neurofibroma known as 'diffuse plexiform' and 'deep nodular' are considered to carry an increased risk of giving rise to MPNST. Individuals with NF1 have an 8 to 13 per cent lifetime risk of MPNST (Evans *et al.*, 2002), which is a significant cause of the decreased life expectancy in the NF1 patient population (Rasmussen *et al.*, 2001). NF1 subjects are also at increased risk of non-nerve-sheath tumours (Mulvihill, 1994) and children in particular are at increased risk for brain stem gliomas, rhabdomyosarcomas, and malignant myeloid leukaemias (Friedman *et al.*, 1999).

The hallmark of NF2 is the development of bilateral vestibular schwannomas. Other commonly associated tumours include schwannomas of other central, spinal, and peripheral nerves, and meningiomas (reviewed in Friedman *et al.*, 1999; Evans *et al.*, 2000; Baser *et al.*, 2003; Evans, 2004; Ruggieri *et al.*, 2005). The majority of patients become completely deaf and can have poor balance, vision, and weakness. Juvenile posterior subcapsular cataract is common. Although NF2 patients are not at increased risk for malignancy, this is a life threatening disorder

Table 1. Features of the neurofibromatoses

Feature	Neurofibromatosis 1 (NF1)	Neurofibromatosis 2 (NF2)	Schwannomatosis <sup>1</sup>
Alternative name:	Peripheral neurofibromatosis; von Recklinghausen neurofibromatosis	Central neurofibromatosis; bilateral acoustic neuroma	Neurilemmomatosis
OMIM accession number <sup>2</sup> :	162200	101000	162091
Mode of inheritance:	Autosomal dominant	Autosomal dominant	Autosomal dominant
Penetrance:	Very high	Very high	Unknown, but reduced compared with NF1 and NF2
Frequency of disorder:	1/3000–1/4000 <sup>3</sup>	1/25,000 <sup>4</sup>	Unknown, but may be as high as NF2
% Sporadic cases:	30–50%	~50%	Unknown, probably > 50%
% Cases with mosaicism:	> 4% <sup>5</sup>	17–28% <sup>6</sup>	Unknown
Gene:	<i>NF1</i>	<i>NF2</i>	Gene not yet identified
Chromosomal location:	17q11.2	22q12.2	22q <sup>7</sup>
Commonly associated tumours:	Neurofibroma, MPNST, optic pathway and brain stem gliomas	Bilateral vestibular schwannomas, schwannomas (without schwannomas of other central and peripheral nerves), meningiomas	Bilateral vestibular schwannomas

<sup>1</sup> Information from recent review MacCollin *et al.*, 2005; <sup>2</sup> Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM>); <sup>3</sup> Friedman *et al.*, 1999; <sup>4</sup> Evans *et al.*, 2005b; <sup>5</sup> About 40 per cent of the estimated 5 to 10 per cent of NF1 contiguous gene deletions are mosaic (Kehrer-Sawatzki *et al.*, 2004); <sup>6</sup> Kluwe *et al.*, 2003; <sup>7</sup> Schwannomatosis locus is not mapped precisely, but is distinct from, and proximal to, the *NF2* gene. MPNST, malignant peripheral nerve sheath tumour.

because of the tumour location and the tendency to develop multiple tumours. The mean age of onset is between 18 and 24 years and the mean age of death is 36 years. The age of onset of symptoms and the age at diagnosis are both predictors of vestibular schwannoma growth rate and risk of death (Baser *et al.*, 2004 and references therein). Diagnostic criteria and a consensus statement on management of the NF2 patient and family were published recently (Baser *et al.*, 2002; Evans *et al.*, 2005a).

### Clinical utility and testing issues

Diagnostic, presymptomatic, prenatal, and preimplantation testing are available for both NF1 and NF2 (consult [www.genetest.org](http://www.genetest.org) and [www.gendia.net](http://www.gendia.net) for information on laboratories that offer testing). The clinical utility of diagnostic testing and presymptomatic testing of at-risk individuals is different for the two disorders. Because a diagnosis of NF1 can be established with high certainty, particularly after 8 years of age, diagnostic and presymptomatic testing is of limited use. However, DNA-based testing is useful to confirm a diagnosis or to identify a mutation in an affected parent, permitting prenatal or preimplantation diagnosis. Furthermore, there has been a recommendation for routine testing to identify carriers of an NF1 contiguous gene deletion (~5 per cent of the patient population; Table 2) for purposes of increased surveillance, as this type of mutation doubles the lifetime risk of MPNST (De Raedt *et al.*, 2003).

Table 2. *NF1* and *NF2* genes and mutations

Feature	NF1 gene	NF2 gene
Gene size; transcript size:	~350 kb; ~11–13 kb <sup>1</sup>	~110 kb; 2 kb <sup>1</sup>
Genbank accession No. (gene; cDNA):	NT_010799; NM_000267	Y18000; NM_000268
Number of exons:	60	17
Protein product (size (kDa); No. of amino acid residues):	Neurofibromin (> 220 kDa; 2818)	Merlin, also known as schwannomin (65 kDa; 595)
Normal protein function:	Tumour suppressor; negative regulator of Ras oncogene <sup>2</sup>	Tumour suppressor; cytoskeletal protein that associates with proteins important in adhesion, signalling, and the cytoskeleton <sup>3</sup>
Types of constitutional mutations (familial and <i>de novo</i> ):	85–90% nonsense, splicing defects, missense; ~5% contiguous gene deletions <sup>4</sup>	Nonsense, splicing defects, multi-exonic deletion, whole gene deletion, 5% missense <sup>5</sup>
Types of mutation in cases of generalized or localized somatic mosaicism:	Contiguous gene deletions <sup>6</sup> ; one case of multiexonic deletion	nonsense, frameshift, splice site <sup>7</sup>
Types of somatic mutation in tumour tissues:	Neurofibroma: splicing defects, LOH <sup>8</sup>	
MPNST: LOH <sup>8</sup> , deletion	Bilateral vestibular schwannomas: nonsense, frameshift, splice site, LOH <sup>8</sup>	

1 Alternative splicing produces transcripts of varying lengths; 2 Reviewed in Dasgupta & Gutman, 2003; 3 Reviewed in McClatchey & Giovannini, 2005; 4 For example, Mattocks *et al.*, 2004; 5 Wallace *et al.*, 2004; Kluwe *et al.*, 2005; 6 Kehrer-Sawatzki *et al.*, 2004; technical difficulties have discouraged the search for subtle intragenic mutations; 7 Kluwe *et al.*, 2005; Moyhuddin *et al.*, 2003; 8 LOH, loss of heterozygosity by mitotic recombination or deletion. MPNST, malignant peripheral nerve sheath tumour.

Less rigorous studies suggest that this subset of patients may be predisposed to childhood overgrowth, early age of onset, and excessive numbers of cutaneous and internal neurofibromas, learning disabilities, vascular anomalies, and astrocytomas (Dorschner *et al.*, 2000; Gutmann *et al.*, 2003; Venturin *et al.*, 2004; Kehrer-Sawatzki *et al.*, 2005; Stephens, 2006a).

*NF1* contiguous gene deletions are recurring 1.4 Mb or 1.2 Mb deletions that span the entire *NF1* gene along with neighbouring genes (Dorschner *et al.*, 2000; Lopez-Correa *et al.*, 2001; Kehrer-Sawatzki *et al.*, 2004; Stephens, 2006a). These recurring deletions arise by recombination between high identity sequence elements that flank the *NF1* gene.

While testing a patient with equivocal findings can be done, it is important to realize that the frequency of positive test results is expected to be quite low. DNA-based clinical testing for NF2 is primarily used for presymptomatic testing of at-risk individuals, typically young children of an affected parent, in conjunction with genetic counselling. An early diagnosis of NF2 improves management, which is primarily surgical and radiological and may improve outcome. For at-risk children who test negative, they and their families will be spared the worry and the expense of periodic diagnostic screening. Correlations between NF2 mutations and phenotype have been described, although they cannot predict the age of onset or the course of disease for an individual patient. Typically constitutional frameshift and nonsense mutations are associated with more severe NF2, defined by earlier age at onset and higher frequency and mean numbers of tumours (Parry *et al.*, 1996; Evans *et al.*, 1998). Constitutional missense and small in-frame mutations are considered to be associated with mild disease, and mutations in splice donor/acceptor sites result in variable clinical outcomes (Evans *et al.*, 1998; Kluwe *et al.*, 1998); although splice site mutations in certain exons are more severe mutations (Baser *et al.*, 2005). Interestingly, certain mutation types are associated with increased non-vestibular nervous system tumours (Baser *et al.*, 2004).

Diagnostic testing to confirm a clinical diagnosis may also be helpful in sporadic NF2 cases in children or adults with equivocal findings or mild disease. Couples seeking prenatal or preimplantation diagnosis for either NF1 or NF2 are advised to seek genetic counselling before pregnancy, as identification of the mutation carried by the affected parent can take weeks or months. A confounding factor in diagnostic testing of either NF1 or NF2 is somatic mosaicism for a post-zygotic mutation (see Table 1 for estimated frequency). Mosaic individuals are always sporadic cases (that is, the first person in their family to have the disease) and carry the mutated gene in only a fraction of their cells, depending upon the developmental interval and cell type in which the mutation occurred (Fig. 1). Therefore, individuals with mosaicism can have localized disease or mild to severe generalized disease; this is likely to be the cause of the clinical entity known as segmental NF1 (Ruggieri & Huson, 2001). Individuals mosaic for an *NF1* or *NF2* mutation have a (50 per cent risk of have an affected child, depending upon the proportion of gametes that carry the mutation. Offspring that do inherit the mutation will have it in every cell of the body and may have more severe disease than the parent. Genetic counselling regarding the clinical and reproductive implications of mosaicism is important (Ruggieri & Huson, 2001).

The profile of constitutional mutations in NF1 and NF2 is similar. Both disorders are both caused by a mutation that inactivates one allele (or copy) of the gene resulting in haploinsufficiency for the protein product of the gene, neurofibromin or merlin, respectively (Table 2). Many types of mutations have been identified that affect the normal gene structure and function at all levels (Table 2, Fig. 2). Thus DNA-based testing for either NF1 or NF2 disease, typically undertaken on peripheral leucocytes, often employs a multipronged approach using molecular techniques that can detect different types of mutation at high frequency. In general, the testing

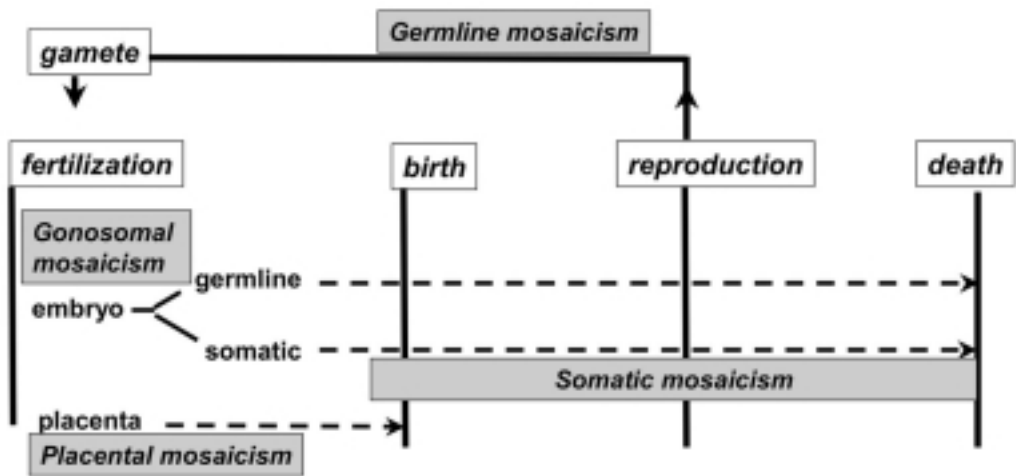


Fig. 1. The types of mosaicism that can occur during the human life cycle. Mutation in *NF1* and *NF2* (and other genes) can occur throughout the life cycle from fertilization to death. Gonosomal mosaicism refers to a mutation that occurs in the early embryo before the split of the germline and somatic progenitors; such individuals are at risk for having the disease and for having an affected offspring. Individuals with only somatic mosaicism may have no, mild, or severe disease depending upon the developmental interval and the somatic cell type; they are not at risk for having affected offspring. In practice, it is impractical or impossible to distinguish between gonosomal and somatic mosaicism unless the patient has an affected child, which would indicate involvement of the germline. Placental and germline mosaicism have no impact on the patient's phenotype but, depending upon the gene involved, may affect fetal development; the consequences of *NF1* or *NF2* placental mosaicism on the fetus are unknown. Germline mosaicism results from a new mutation in a germ cell or progenitor germ cell; the carrier will not develop the disease, but their offspring are at risk of inheriting a gamete carrying a mutation and being affected.

methodologies employ either direct sequence analysis of amplified products of each exon and the associated intronic regions or mutation scanning methodology (for example, denaturing high performance liquid chromatography, premature protein termination, heteroduplex analysis) that detects putative mutations in amplified products from genomic or cDNA templates. These are subsequently confirmed by direct DNA sequence analysis or other appropriate method. The detection of *NF1* contiguous gene deletions typically involves fluorescence *in situ* hybridization, while smaller multiexonic deletions, typical of *NF2*, are often detected by a type of quantitative gene dosage assay. Routine cytogenetic analysis has a very low sensitivity for detecting *NF1* or *NF2* mutations. A recent review of available assays used in *NF1* and *NF2* testing has been published (Stephens, 2006b). The choice of assay and testing laboratory depends upon the reason for referral and the detection rates for different mutation types and mutation. Although published reports may give high detection rates for a specific protocol, it is important to realize that detection rates – particularly for mutation scanning protocols – will be laboratory-specific owing to the degree of optimization of a specific technique. The clinician is advised to survey testing laboratories before submitting samples.

DNA-based diagnosis of *NF2*, but not *NF1*, can often be facilitated by analysis of unused pathology tissue after surgical removal of a bilateral vestibular schwannoma. These tumours develop from a progenitor Schwann cell that carries a somatic inactivating mutation in the single remaining *NF2* gene. Thus these cells have no functional merlin. Because these tumours are clonal with minimal cellular admixture, *NF2* mutations can be detected at high frequency.

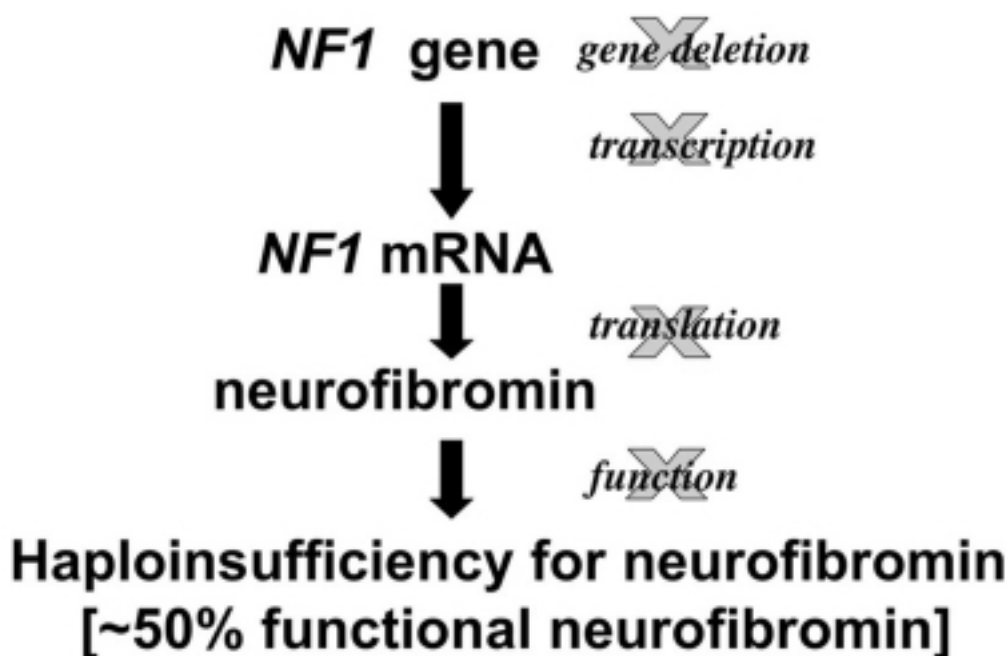


Fig. 2. The consequences of *NF1* gene mutation. Different types of *NF1* mutation affect the gene structure and function at all levels from deletion of the entire gene to inhibiting transcription or transcript stability, affecting translation or inactivating, or altering the normal function of the protein. The result of all these mutations is haploinsufficiency for neurofibromin. Although this schematic is for *NF1*, the same is true for the *NF2* gene and its protein product merlin.

This is most useful in cases where a mutation is not detected in primary leucocytes of a patient in whom the clinical manifestations suggest somatic mosaicism, or where normal tissue is unavailable (Kluwe *et al.*, 2003; Moyhuddin *et al.*, 2003; Wallace *et al.*, 2004). For *NF2* patients undergoing surgery, it is advisable to arrange to have a portion of the tumour frozen and saved, as it may be valuable for future mutational analysis for the benefit of the patient or their relatives. Although neurofibromas and MPNST of *NF1* patients also arise from progenitor cells with both copies of the *NF1* gene inactivated, these tumours are typically not useful tissue for mutational analyses because the tumour is composed of numerous other cell types that compromise mutation detection.

Linkage testing for *NF1* and *NF2* may be the quickest, most economical test for at-risk individuals and fetuses of families that fulfil the testing criteria. This is an indirect form of testing that does not identify the specific mutation in the gene but tracks the inheritance of the mutant *NF1* or *NF2* allele in members of a family (Fig. 3). Multiple family members whose *NF1* (or *NF2*) disease status is unambiguous must participate in the testing process. Prenatal testing of the example in Fig. 3 would determine if the fetus inherited the at-risk haplotype R. Linkage testing is compromised by somatic mosaicism and this possibility must be evaluated carefully before embarking on this form of testing (Fig. 3). Additional issues related to test interpretation and laboratory issues related to clinical testing for *NF1* and *NF2* have been reviewed recently (Stephens, 2006b).

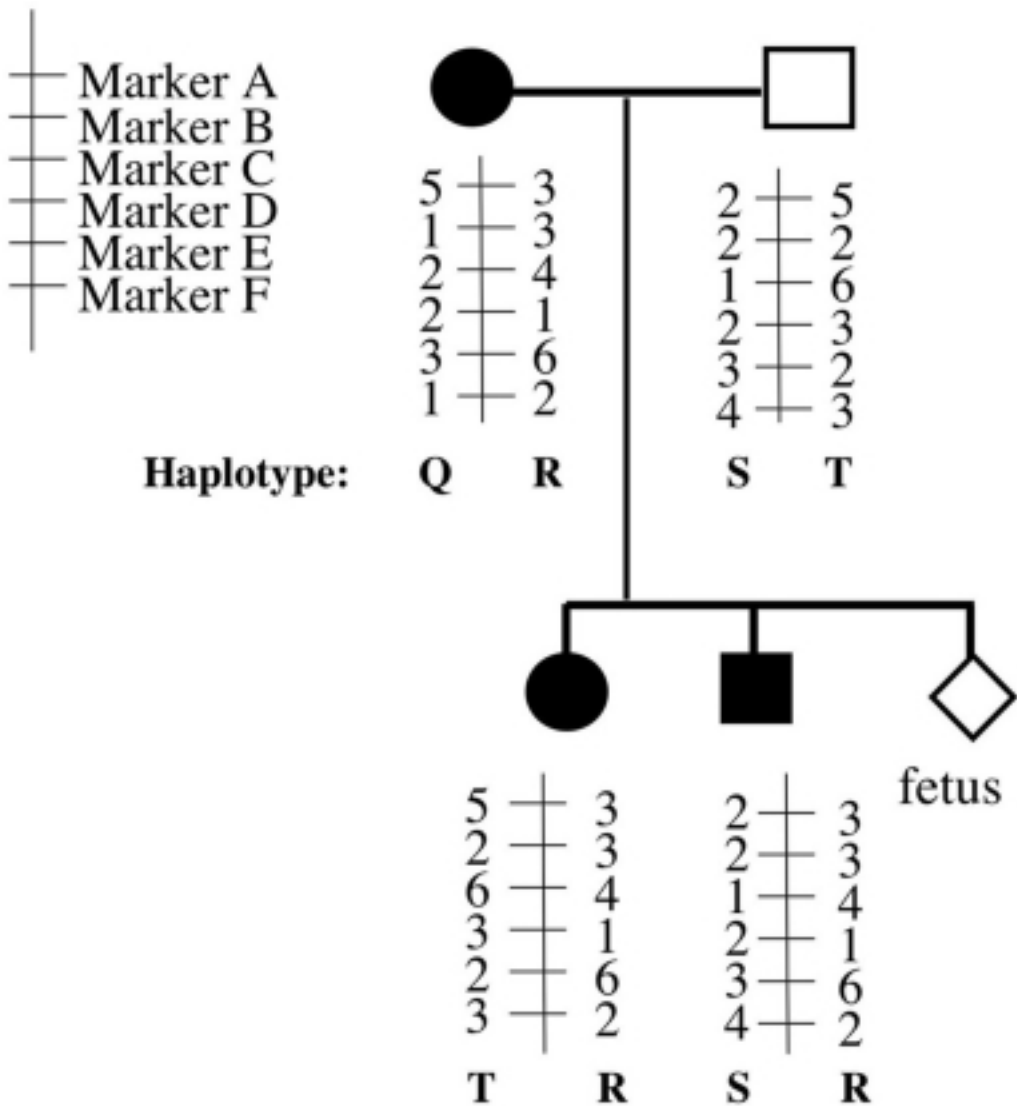


Fig. 3. Example of linkage testing for prenatal diagnosis. The schematic shows a generic example of linkage testing in which markers A-F are polymorphic sites at known positions in, or very close to, the gene of interest. The genotypes at each marker are determined in multiple family members of known disease status. The arrangement of genotypes in this segment of the chromosome is known as a haplotype. In this example, the at-risk haplotype carrying the mutated gene is R; testing the fetus will determine if it has inherited the R haplotype and therefore the disease. Gonosomal mosaicism may compromise linkage testing: if the mother is a gonosomal mosaic, then a fraction of her R chromosomes would carry a mutant gene and a fraction would have a normal gene. Therefore, if the fetus inherited the R haplotype, one could not predict the disease status. Circles, female; squares, male; filled symbols, affected; empty symbols, unaffected; diamond, foetus.

## Conclusions

In the past 5 years, significant advances have been made in understanding the types of mutation that inactivate the *NF1* and *NF2* genes and their clinical consequences. This has led to the development and availability of sensitive and specific DNA-based tests for diagnostic, pre-symptomatic, prenatal, and preimplantation diagnosis of the NF1 and NF2 disorders. Already these tests have altered clinical diagnosis, management, and outcome for at least a subset of affected patients. Future advances are expected to provide new correlations between genotype and management, and possibly therapeutic, decisions for clinicians caring for individuals affected with these disorders.

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**Chapter 20.**

**The Neurofibromatoses**

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Neurofibromatosis 1 and neurofibromatosis 2 are two distinct genetic disorders that predispose to the development of tumors primarily of the nervous system (Table 20-1)<sup>1</sup>. A recently recognized third form of neurofibromatosis, known as schwannomatosis,<sup>2</sup> is not included in this review as molecular genetic testing is unavailable for this disorder.

## **NEUROFIBROMATOSIS (NF1)**

### **MOLECULAR BASIS OF DISEASE**

NF1 is an autosomal dominant progressive disorder with high penetrance but extremely variable expressivity (reviewed in Ref.<sup>1,3,4</sup>). The cardinal features are café au lait macules, intertriginous freckling, Lisch nodules, and multiple neurofibromas, although numerous other features and complications are not uncommon. Criteria for a diagnosis of NF1 established in 1987 by a consensus meeting of The National Institutes of Health diagnostic criteria are widely used.<sup>5</sup> Neurofibromas are benign nerve sheath tumors that arise on peripheral nerves. Cutaneous neurofibromas develop in virtually all cases of NF1, typically appear in second decade of life, grow slowly, increase in number with age, and considered at low risk for transformation to a malignant peripheral nerve sheath tumor (MPNST; previously known as neurofibrosarcoma). However, diffuse plexiform neurofibromas and deep nodular plexiform neurofibromas are considered at increased risk for transformation to MPNST. Individuals affected with NF1 have a lifetime risk for MPNST of 8-13%.<sup>6</sup> Other neoplasms epidemiologically-associated with NF1 include medulloblastoma, pheochromocytoma, astrocytoma, adenocarcinoma of the ampulla of Vater. Primarily children affected with NF1 are at increased risk for optic pathway gliomas and brainstem gliomas, rhabdomyosarcomas, and malignant myeloid leukemias. NF1 patients are also at increased risk for a second malignancy, some of which may be treatment-related.

NF1 is caused by inactivating mutations of one copy of the *NF1* gene resulting in haploinsufficiency for the gene product neurofibromin (Table 20-1). About 85-90% of constitutional

mutations are nonsense, splicing, and missense; they are distributed throughout the gene although some exons appear to be mutation-rich (Fig. 20-1). An estimated 5 % of mutations are large contiguous gene deletions typically of 1.4 Mb that delete one entire *NF1* allele (reviewed in Ref<sup>7</sup>). About one-half of cases are familial (inherited from an affected parent) and one-half are sporadic resulting from a *de novo NF1* mutation. An unknown fraction of sporadic cases are due to post-zygotic mutation of the *NF1* gene, which complicates mutation detection and counseling issues. Neurofibromin functions as a negative regulator of the Ras oncogene by stimulating the conversion of active GTP-bound Ras to the inactive GDP-bound form by hydrolyzing GTP. Biochemical, cell culture, and genetic studies in both NF1 patients and mouse models are consistent with a model whereby a somatic mutation inactivates the remaining functional *NF1* gene (leading to increased activated Ras) in a progenitor Schwann cell as an early, probably initiating, event in the development of neurofibromas (reviewed in Ref<sup>8</sup>). Biallelic inactivation of *NF1* also occurs in other types of progenitor cells that give rise to NF1-associated tumors such as glioma and myeloid malignancies.

## CLINICAL UTILITY OF TESTING

A diagnosis to NF1 can nearly always be made based on clinical findings, particularly after 8 years of age. Clinical DNA-based testing is available from many licensed diagnostic laboratories (<http://www.genetests.org/>). Testing is not typically used for diagnostic purposes, but can be useful for confirming a clinical diagnosis, reproductive counseling, prenatal or preimplantation diagnosis. Blanket recommendations for diagnostic testing for NF1 cannot be made because the sensitivity of making a clinical diagnosis is very high and the sensitivity of molecular testing is not 100%. Furthermore, benefits of diagnostic testing are subjective and may differ from family to family. Early planning is necessary for couples considering prenatal diagnosis of amniocytes or chorionic villus tissue or preimplantation diagnosis. These tests are available only in cases where the pathogenic germline mutation (or the predisposing haplotype in the case of linkage testing) has been identified previously in an affected parent, a process that can require weeks or months.

The primary genetic counseling issue related to molecular testing of NF1 is the inability to predict the severity or course of the disorder in a patient or fetus. Even among family members who carry the same *NF1* mutation, there can be considerable variation in clinical manifestations and complications. For the majority of cases, there is no correlation between genotype and phenotype. For the ~5% of individuals that carry a constitutional *NF1* deletion (most commonly 1.4 Mb) there is a two-fold increased lifetime risk of MPNST<sup>9</sup>, a predisposition to childhood overgrowth<sup>10</sup>, an early age of onset and excessive numbers of cutaneous neurofibromas, a high load of internal neurofibromas, learning disabilities, vascular anomalies, and astrocytomas (Ref.<sup>7, 11-14</sup> and references therein). A recommendation for routine testing for NF1 microdeletion has been proposed with followup for increased suspicion for MPNST<sup>9</sup>. *NF1* testing may be useful to confirm a diagnosis in a patient with equivocal findings, such as child who has a few café au lait macules and carries a presumptive diagnosis of NF1. However, in this instance it is important to realize that the sensitivity and specificity of testing patients who do not fulfill the NIH diagnostic criteria for a diagnosis of *NF1* is unknown, but is likely to be quite low. For unaffected parents of a child with sporadic NF1, recurrence risk is a concern. Although thought to be rare, germline mosaicism has been reported in an asymptomatic parent of a child with sporadic NF1.<sup>15</sup> Therefore, there is a small, but unknown, increased risk of recurrence even if the child's pathogenic mutation is not detected in the genomic DNA from parental leukocytes. Although the frequency is unknown, sporadic NF1 cases with post-zygotic mutations resulting in somatic mosaicism may not be as rare as once thought. One study suggests that among NF1 microdeletion cases, the frequency of somatic deletion may be very high (~40%).<sup>16</sup> Assuming 10% NF1 cases are microdeletions, a frequency of 4% mosaicism is expected in the general NF1 population. This is certainly an underestimate as it does not consider mosaicism for intragenic *NF1* mutations. Mosaic individuals carry the *NF1* mutation in only a fraction of their cells, depending upon the developmental interval and the cell type in which the mutation occurred. The phenotype of mosaic individuals ranges from localized (segmental) disease to mild or severe generalized disease<sup>16, 17</sup>. The sensitivity of mutation detection may be lower due to an increased signal to noise ratio, i.e., a low level of a mutant allele in a background of two normal *NF1* alleles. Offspring that

inherit an NF1 mutation from a parent with mosaicism, however, will have a constitutional *NF1* mutation and may have more severe disease than their mosaic parent. Genetic counseling regarding the clinical and reproductive implications of NF1 mosaicism is highly recommended.<sup>17</sup>

#### AVAILABLE ASSAYS

Mutation of the *NF1* gene is the only known cause of the disorder. Molecular tests for diagnostic, prenatal, and preimplantation diagnosis are available. The choice of assay and testing laboratory depends upon the reason for referral and mutation types and detection rates of their assay(s).

Fluorescent in situ hybridization (FISH; see Chapter 2) with *NF1* probes of either metaphase or interphase white blood cells is the optimal test to rule out/confirm the ~5% of cases due to a submicroscopic NF1 microdeletion<sup>18</sup> (Figure 20-1). In the future, a first-tier test may employ an *NF1* deletion junction-specific PCR assay.<sup>19</sup> The recent availability of high-resolution genomic microarrays of the NF1 deletion region will facilitate clinical testing by array-CGH,<sup>20</sup> which may become clinically important in the future if deletions involving a subset of genes predispose to certain manifestations. The sensitivity of deletion-specific PCR and array-CGH assays to detect low level *NF1* deletion mosaicism will need to be determined. Routine cytogenetic analysis is of limited clinical utility as the *NF1* microdeletions are submicroscopic and translocation/rearrangement involving *NF1* are extremely rare.

Linkage analysis is an indirect test that tracks the inheritance of the mutant *NF1* allele in members of a family. This may be the quickest, most economical NF1 test for at-risk individuals and fetuses of families that fulfill the testing criteria. The primary requirement is the availability and cooperation of multiple family members whose NF1 status is known by detailed clinically evaluation. The availability of multiple *NF1* intragenic polymorphic markers facilitates identification and tracking of the predisposing haplotype in a family and the specificity of linkage testing.

Efficient detection of subtle intragenic *NF1* gene mutations, for purposes of diagnostic testing or mutation typing for prenatal or preimplantation diagnosis, is complicated by the large number of exons and size of the gene (Table 20-1), variation in type and distribution of mutations, and large fraction of

private mutations. About 70-80% of mutations result in a premature translation termination codon, with nonsense and splicing defects being the most common.<sup>21</sup> These mutations can be detected by the protein truncation test (PTT) (see Chapter 2), which detects truncated neurofibromin polypeptides synthesized by *in vitro* translation of multiple overlapping *NF1* cDNA segments. A detection rate of about 80% can be attained with an optimized PTT protocol (see below). The majority of such mutations are private to each individual/family, although there are recurrent mutations that may account for, at most, a few percent of cases (Figure 20-1). About 10% of *NF1* mutations are missense or in-frame insertions/deletions of a few nucleotides,<sup>21, 22</sup> some of which show clustering (Figure 20-1). Their identification requires direct sequence analysis of *NF1* exons and splice junctions in genomic DNA or of cDNA segments.

Prospective testing of *NF1* subjects by direct genomic sequence analysis revealed a detection rate of 89%, which is more streamlined than PTT testing and allows for automation.<sup>23</sup> Various mutation scanning techniques of *NF1* genomic or cDNA are also employed by some testing laboratories. Techniques may include denaturing high performance liquid chromatography (DHPLC), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), and/or heteroduplex analysis (HA) (see Chapter 2). Although reports in the literature of a high detection rates by DHPLC (72-95%),<sup>24,25</sup> it is important to realize that the detection rates for mutation scanning protocols will be laboratory-specific due to the degree of optimization of the specific technique. A survey of testing laboratories is recommended prior to sample submission. DHPLC has the advantages of using genomic DNA and high throughput capability compared to the cDNA/gel-based PTT. However, a recently reported high-throughput PTT may be available for clinical testing in the future.<sup>26</sup>

## INTERPETATION OF TEST RESULTS

The detection of a truncated neurofibromin polypeptide by PTT can result in false positives.<sup>21</sup> High specificity requires identifying the underlying mutation at the genomic DNA and/or cDNA levels

since false positives can arise during sample handling (see below). The interpretation of missense and subtle in-frame alterations as pathogenic mutations versus neutral polymorphisms is complicated by the lack of a functional assay for neurofibromin. Apparent recurrence of a putative mutation requires careful study of the literature, as not all *NF1* mutational analyses sequenced the entire gene. No comprehensive *NF1* mutation database is available, however some mutations have been submitted to the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>) and the largest *NF1* database is actively managed and analyzed by Jan Friedman (<http://www.medgen.ubc.ca/friedmanlab/>). Although most likely rare, affected family members with different, independent *NF1* inactivating have been reported,<sup>27</sup> presumably a reflection of the high mutation rate of the gene ( $\sim 10^5$ /gamete/generation). The interpretation of FISH with *NF1* probes can be complicated by mosaicism for an *NF1* microdeletion; an appropriate number of cells must be analyzed.<sup>16</sup> The frequency of mosaicism for an *NF1* mutation is not known, however this is likely the underlying mechanism for patients with segmental or localized signs of the disorder.

## LABORATORY ISSUES

Optimal detection of mutations that predict a truncated neurofibromin polypeptide occurs when the nonsense-mediated decay pathway is at least partially inhibited, thereby increasing the ratio of mutant transcripts with a premature termination codon to normal transcripts. A protein synthesis inhibitor, such as puromycin, in the culture medium is effective for EBV-transformed lymphoblasts or phytohemagglutinin-stimulated primary lymphocytes.<sup>21, 28</sup> Furthermore, blood handling and shipping protocols must be used to reduce false positives in PTT resulting from environmental effects such as cold shock<sup>29</sup> or delay in mRNA isolation.<sup>21, 28</sup> There is no standardized proficiency program of interlaboratory comparison for *NF1* testing; performance assessment must be conducted by participation in ungraded proficiency survey programs, split sample analysis with other laboratories, or other suitable and documented means. There are no Food and Drug Administration-approved *NF1* testing kits, probes, or controls. Intronic primers for amplification of *NF1* exons and associated splice junctions that apparently

do not co-amplify the *NF1* pseudogene fragments have been reported.<sup>23,30</sup> Two other factors require consideration during test development and interpretation. Reports in support of,<sup>31</sup> and in opposition to,<sup>32</sup> an apparent tandem duplication of the *NF1* gene region have been published. In addition, transcriptional activity from *NF1* pseudogenes or pseudogene fragments has been reported.<sup>33</sup> Some issues related to NF1 testing have been reviewed recently.<sup>34</sup>

## NEUROFIBROMATOSIS 2 (NF2)

### MOLECULAR BASIS OF DISEASE

The development of bilateral vestibular schwannomas is a hallmark of NF2. Other commonly-associated tumors include schwannomas of other central, spinal, and peripheral nerves and meningiomas (reviewed in Ref<sup>1,35-38</sup>). This is a life-threatening disorder due to the location of the tumors, along with the propensity for development of multiple tumors. Most patients become completely deaf and can have poor balance, vision and weakness. The mean age of onset is 18-24 years and the mean age of death is 36 years. The age at onset of symptoms and age at diagnosis are predictors of vestibular schwannoma growth rates and risk of death (Ref.<sup>39</sup> and references therein). Ependymomas and astrocytomas occur less frequently and are usually indolent CNS tumors. Patients affected with NF2 are at minimal increased risk for malignancy. Juvenile posterior subcapsular cataract is a common non-tumor manifestation. The disorder may be under-diagnosed in children who present with ocular and skin manifestations. Early diagnosis improves management, which is primarily surgical and radiological. Modifications to the criteria for a diagnosis of NF2, initially established by the 1987/1991 National Institute of Health Consensus Conference, have been proposed to increase the specificity.<sup>40</sup> A consensus statement on management of the NF2 patient and family was recently published.<sup>41</sup>

NF2 is caused by haploinsufficiency for the tumor suppressor merlin (also known as schwannomin), the protein product of the *NF2* gene (Table 20-1). About one-half of patients are the first case of NF2 in the family. These sporadic cases result from *de novo* mutation of the *NF2* gene, a

significant fraction of which are postzygotic mutations that result in mosaicism. The majority of constitutional mutations is private, predicts the truncation of merlin, and is distributed throughout the gene (see below). In NF2 patients, a vestibular schwannoma develops from a progenitor Schwann cell that carries a somatic inactivating mutation in the single remaining *NF2* gene. Merlin is a protein of the cytoskeleton whose normal function remains to be determined, although it is known to associate with transmembrane proteins important in adhesion, proteins involved in signaling pathways, and cytoskeletal proteins (reviewed in Ref. <sup>42</sup>).

#### CLINICAL UTILITY OF TESTING

DNA-based clinical testing for NF2 is available ([www.genetests.org](http://www.genetests.org)) and primarily used for presymptomatic testing of at-risk individuals, typically young children of an affected parent. An early diagnosis of NF2 may improve outcome and at-risk children who did not inherit the *NF2* mutation can be spared worry, costly brain imaging, and audiologic screening. Genetic counseling is recommended prior to testing presymptomatic at-risk children. Testing is also useful to confirm a clinical diagnosis, which may be most helpful in sporadic cases of NF2, particularly children who present with ocular or skin manifestations or adults with equivocal findings or mild disease. Some of these cases may be mosaic for an *NF2* mutation, as the estimated frequency of mosaicism is high (16.7 – 24.8% of sporadic cases).<sup>43</sup> Genetic counseling regarding the clinical and reproductive implications of NF2 mosaicism is recommended.<sup>17</sup> Testing is also useful for reproductive counseling and prenatal or preimplantation diagnosis.<sup>44</sup> Prenatal diagnosis of NF2 using amniocytes or chorionic villus tissue is available only in cases where the pathogenic NF2 germline mutation (or predisposing haplotype in the case of linkage testing) has been identified previously in an affected parent. For preimplantation genetic diagnosis, the specific parental NF2 mutation must be known. Pre-pregnancy planning is important for couples considering prenatal diagnosis or preimplantation diagnosis. For sporadic NF2 patients undergoing surgery, it is advisable to freeze a portion of the tumor, which may be valuable at a later date for mutation identification if the patient is mosaic.

The primary genetic counseling issues regard predicting the course of the disorder and recurrence risks. There are genotype/phenotype correlations, but they cannot predict the age of onset or the course of disease for an individual patient. About 50% of *NF2* mutations are nonsense or frameshift, with about 24% splice site, 11-30% submicroscopic deletion, and 5% missense.<sup>45</sup> Typically, constitutional frameshift and nonsense mutations are associated with more severe *NF2*, defined by earlier age at onset and higher frequency and mean number of tumors.<sup>46, 47</sup> Constitutional missense and small in-frame mutations are considered to be associated with mild disease<sup>47</sup> and mutations in splice donor and acceptor sites result in variable clinical outcomes.<sup>48</sup> Interestingly, individuals with *NF2* splice sites mutations in exons 1-5 had an earlier age at onset and greater numbers of intracranial meningiomas compared to those with splice site mutations in exons 11-15<sup>45</sup> (Figure 1B). The type of constitutional *NF2* mutation is also correlated with the number of *NF2*-associated non-vestibular nervous system tumors including intracranial meningiomas, spinal tumors, and peripheral nerve tumors.<sup>39</sup> Individuals with constitutional nonsense or frameshift *NF2* mutations had significantly more such tumors than individuals carrying missense, splice-site, or deletion mutations or somatic mosaicism.

Recurrence risks for asymptomatic parents of an affected child are unknown, but are somewhat greater than the population risk due to the possibility of germline mosaicism in a parent.<sup>49</sup> For mosaic patients, the risk of transmitting *NF2* to offspring is  $\leq 50\%$ , depending upon the proportion of gametes that carry the *NF2* mutation.<sup>17</sup> Offspring that do inherit the mutation however, will have a constitutional *NF2* mutation and may have more severe disease than their mosaic parent. Testing asymptomatic parents of a child with *NF2* has the potential to identify a mosaic mutation.

#### AVAILABLE ASSAYS

Mutation of the *NF2* gene is the only known cause of the disorder. Linkage analysis is clinically available for at-risk individuals and fetuses with multiple family members of unambiguous clinical status regarding *NF2* disease who are willing to participate in the testing process. The availability of highly informative intragenic *NF2* intragenic polymorphisms increases the specificity of this method. For

certain families, linkage analysis will be the most cost and time effective test that gives a definitive diagnosis. It can sometimes be an option when mutation scanning or sequencing test results are negative. See interpretations below for cautions regarding linkage test interpretation.

Identifying an *NF2* mutation typically requires a multipronged testing protocol due to the high frequency of private constitutional mutations, the high frequency of postzygotic mutations, the different types of *NF2* mutations, and the distribution of mutations throughout the gene. Wallace et al <sup>50</sup> describe a comprehensive testing service that includes four PCR reactions using a meta-PCR technique to link the amplicons into chimeric concatemers for direct sequencing, gene dosage PCR for deletions, LOH studies and subsequent sequencing of the gene in tumor tissue. In prospective studies, this approach yielded an 88% detection rate in familial NF2 cases and a 59% detected rate in sporadic NF2 cases. Direct sequencing or exon scanning techniques (e.g., SSCP, TGGE, and HA (see Chapter 2)), of DNA from peripheral leukocytes followed by direct sequencing to identify the underlying *NF2* mutation, generally have a lower detection rate<sup>46, 47, 51-53</sup> The detection rate of either sequencing or exon scanning methods is significantly lower (34-51%) in sporadic cases due in part to the high frequency of post-zygotic *NF2* mutations, which can be masked by the presence of normal alleles.<sup>47, 48, 51, 53</sup> The mutation detection rate of mosaic cases can be increased significantly by analysis of tumor tissue (see below).

Because schwannomas are clonal tumors with minimal cellular admixture, *NF2* mutations can be detected at high frequency in tumor tissue. Testing of tumor tissue is available clinically (<http://www.geneclinics.org/>) and is most useful in cases where a mutation is not detected in primary lymphoblasts, where clinical manifestations are suggestive of somatic mosaicism, or where constitutional tissue is unavailable.<sup>43, 50, 53</sup> Moyhuddin et al<sup>53</sup> nearly doubled the mutation detection rate among mosaic cases using vestibular schwannoma tissue versus peripheral leukocytes. Mutations are likely to be germline (rather than somatic) if the identical mutation is detected in two or more pathologically or anatomically distinct tumors or if a tumor shows loss of heterozygosity (LOH) for *NF2* intragenic/flanking loci, while constitutional tissue is heterozygous at these loci. Mutational analysis of

tumor tissues is expected to have the greatest sensitivity for *NF2* somatic mosaic mutations<sup>43</sup> and sporadic cases with negative results from mutation scanning or sequencing tests.<sup>54</sup>

Efficient detection of the 11-30% of constitutional *NF2* deletions (typically multiexonic in nature) has employed numerous techniques including FISH, various gene dosage polymerase chain reaction (PCR) assays, multiplex ligation-dependent probe amplification (MPLA), and high-resolution genomic arrays.<sup>53, 55-57</sup>

Note that for mutation scanning tests and deletion-detection assays, detection rates will be laboratory-specific due to the varying degrees of optimization of the technique; a survey of testing laboratories is recommended prior to sample submission.

## INTERPRETATION OF TEST RESULTS

Interpretation of the results of exon scanning tests requires identifying the underlying *NF2* mutation at the genomic DNA and/or cDNA levels to avoid false positives. Functional assays for merlin have been developed that can provide insight into the interpretation of missense and subtle in-frame alterations as pathogenic mutations versus neutral polymorphisms;<sup>58-60</sup> however, such assays may not be part of a clinical testing protocol. An international *NF2* mutation database is available <http://uwcmml1s.uwcm.ac.uk/uwcm/mg/nf2/> and this site also recognizes the United Kingdom population-based registry. Some mutations are also detailed in the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>). Somatic mosaicism or *NF2* gene deletions must be considered in patients that have a negative mutation test using DNA from peripheral leukocytes, regardless of the severity of their manifestations. The risk of recurrence from mosaic parent to offspring is considered very low if an *NF2* mutation cannot be identified in the parent.<sup>53</sup> *NF2* linkage tests should consider excluding the first affected member in a family; if they are mosaic the linkage results will be misleading in the next generation.<sup>61</sup> For similar reasons, linkage analysis for presymptomatic testing of subjects in this “next” generation should be performed with caution.

## LABORATORY ISSUES

There is no standardized proficiency program of interlaboratory comparison for *NF2* testing; performance assessment must be conducted by participation in ungraded proficiency survey programs, split sample analysis with other laboratories, or other suitable and documented means. There are no Food and Drug Administration-approved *NF2* testing kits, probes, or controls. Direct gene sequencing may not be the optimal test to detect *NF2* mosaic mutations in lymphoblasts, as reliable detection of a low level point mutation will be difficult. Exon scanning techniques that are semi-quantitative, such as TGGE, will detect relative intensity differences between heteroduplexes and homoduplexes that suggest possible mosaicism.<sup>51</sup> Depending upon age, fixation, and storage conditions, some tumors may not yield nucleic acid of sufficient quality for mutational analysis.

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Table 20-1. Comparison of features of the NF1 and NF2 disorders.

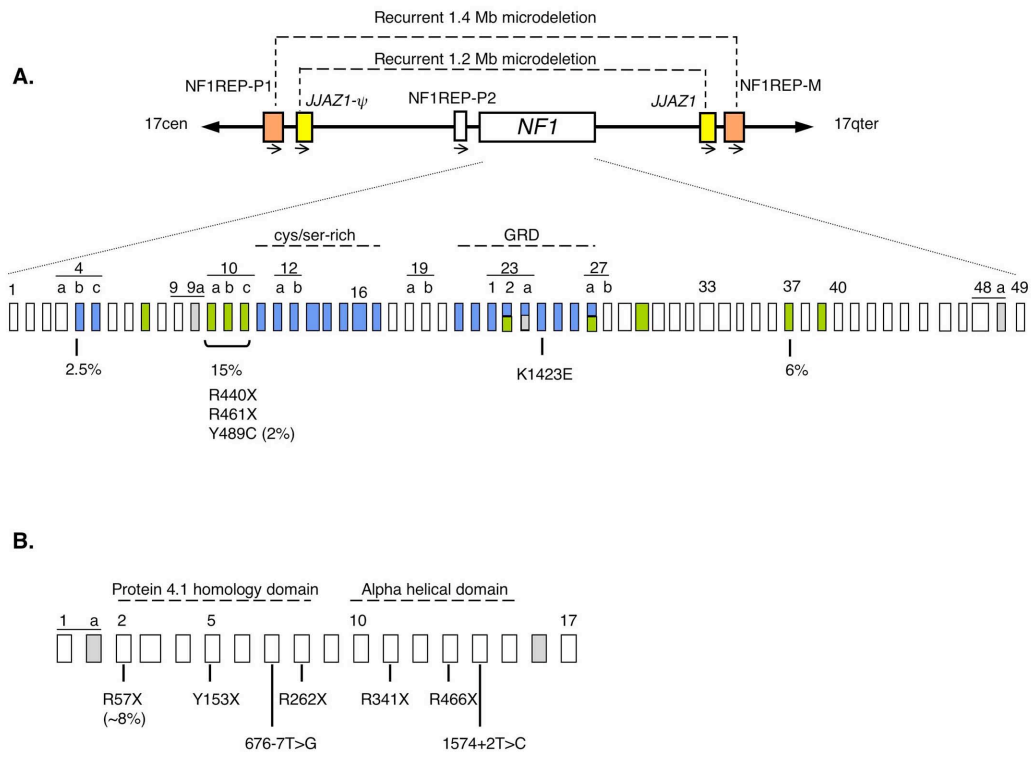
Feature	Neurofibromatosis 1 (NF1)	Neurofibromatosis 2 (NF2)
Alternate name	peripheral neurofibromatosis; von Recklinghausen neurofibromatosis	central neurofibromatosis; bilateral acoustic neuroma
OMIM accession number <sup>1</sup>	162200	101000
Mode of inheritance	autosomal dominant	autosomal dominant
Frequency of disorder	1/3000–1/4000	1/25,000
Fraction of sporadic cases	30-50%	~50%
Gene symbol	<i>NF1</i>	<i>NF2</i>
Chromosomal location	17q11.2	22q12.2
Gene size; transcript size	~350 kb; ~11-13 kb <sup>2</sup>	~110 kb; 2 kb <sup>2</sup>
Genbank accession no. (gene;cDNA) <sup>3</sup>	NT_010799; NM_000267	Y18000; NM_000268
Number of exons	60	17
Tissue expression pattern	Widely expressed	Widely expressed
Protein product (size (kD); # residues)	Neurofibromin (>220 kD; 2818)	merlin, also known as schwannomin (65 kD; 595)
Normal functions of protein	tumor suppressor; negative regulator of ras oncogene	tumor suppressor; associates with proteins of the cytoskeleton
Commonly-associated tumors	Neurofibroma, MPNST, optic pathway and brainstem gliomas	Bilateral vestibular schwannomas, schwannomas of other central and peripheral nerves, meningiomas
Animal models	mouse, fruit fly	mouse, fruit fly

<sup>1</sup>Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM>).<sup>2</sup>Alternative splicing produces transcripts of varying lengths.<sup>3</sup>See Gene Lynx Human (<http://www.genelinx.org/>) for a compilation of, and hyperlinks to, gene, protein structure, and genomic resources.

## FIGURE LEGENDS

Figure 20-1. *NF1* and *NF2* genes: Genomic structure and mutations. A). At the top is a schematic of the *NF1* gene region at chromosome segment 17q11.2. The 350 kb *NF1* gene is flanked by two different sets of directly-oriented paralogs. The 51 kb paralogs NF1REP-P1 (previously termed NF1REP-P) and NF1REP-M (orange boxes) and the 46kb *JJAZ1* gene and pseudogene (- $\psi$ )(yellow boxes). Homologous recombination between NF1REP elements results in a recurrent 1.4Mb microdeletion, while recombination between the *JJAZ1* paralogs results in a recurrent 1.2 Mb microdeletion.<sup>11, 16</sup> NF1REP-P2 is a partial element with a limited role in mediating *NF1* microdeletions. The 60 exons of the *NF1* are represented by boxes (not to scale) and exon numbering is sequential except as indicated. The GRD (exons 21-27a) and a cysteine/serine-rich domain with 3 cysteine pairs suggestive of ATP binding (exons 11-17) are indicated. Grey boxes indicated alternatively spliced exons that vary in abundance in different tissues. Mutations have been identified in virtually every exon. Exons where mutations are apparently in greater abundance than expected are indicated (green boxes).<sup>21, 22, 25</sup> In one study, exons 7, 10a,b,c, 23-2, 27a, 29, 37 and accounted for 30% of mutations, 15 of which were in exons 10a,b,c, which harbor 3 recurrent mutations, including Y489C, which alone may account for ~2% of mutations.<sup>21</sup> Blue boxes indicate exons that had clusters of missense and/or single base/codon deletion mutations.<sup>22</sup> Some of the recurrent, although still infrequent, mutations are given below the exons. B) The 17 exons of the *NF2* gene are represented by boxes (drawing not to scale) and the exon numbering is sequential. The Protein 4.1-homology domain thought to mediate binding to cell surface glycoproteins (exons 2-8), the  $\alpha$ -helical domain (exons 10-15), and the unique C-terminus (exons 16-17) are shown. Grey boxes indicated alternatively spliced exons; the inclusion of exon 16 creates a alternate termination codon resulting in a slightly truncated protein. Mutations have been identified involving each exon except for 16. Selected recurrent mutations found in a limited survey of references cited in the text are indicated. In several studies, R57X occurred in 8% of familial constitutional mutations.

Figure 20-1.



# **Interstitial Uniparental Isodisomy at Clustered Breakpoint Intervals is a Frequent Mechanism of *NF1* Inactivation in Myeloid Malignancies**

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Running head: Interstitial isodisomy in myeloid malignancies

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## ABSTRACT

To identify the mechanism of loss of heterozygosity (LOH) and potential modifier gene(s), we investigated the molecular basis of somatic *NF1* inactivation in myeloid malignancies from ten children with neurofibromatosis type 1. Loci across a minimal 50 Mb region of primarily the long arm of chromosome 17 showed LOH in 8 cases, whereas a <9 Mb region of loci flanking *NF1* had LOH in the remaining 2 cases. Two complementary techniques, quantitative PCR and fluorescence in situ hybridization (FISH), were employed to determine if the copy number at loci that showed LOH was one or two (i.e., deleted or isodisomic). The 2 cases with LOH limited to <9 Mb were intrachromosomal deletions. Among the 8 leukemias with 50 Mb LOH segments, four had partial uniparental isodisomy and four had interstitial uniparental isodisomy. These isodisomic cases showed clustering of the centromeric and telomeric LOH breakpoints. This suggests that the cases with interstitial uniparental isodisomy arose in a leukemia-initiating cell by double homologous recombination events at intervals of preferred mitotic recombination. Homozygous inactivation of *NF1* favored outgrowth of the leukemia-initiating cell. Our studies demonstrate that LOH analyses of loci distributed along the chromosomal length along with copy-number analysis can reveal novel mechanisms of LOH that may potentially identify regions harboring “cryptic” tumor suppressor or modifier genes whose inactivation contribute to tumorigenesis.

## INTRODUCTION

Tumor suppressor gene (TSG) inactivation commonly occurs by sequential somatic inactivation of both alleles or, in individuals who inherit a germline mutation, by a somatic mutation in the single normal homolog. In both groups of patients, somatic inactivation is frequently associated with loss of heterozygosity (LOH) at the TSG locus and at flanking loci.<sup>1,2</sup> Defining the minimal chromosomal region with LOH in a collection of tumors has been a successful strategy for mapping and cloning TSGs. LOH can occur by multiple mechanisms as demonstrated by the extensive analyses of retinoblastomas, which identified *RBI* intragenic deletions, segmental chromosomal deletions, loss of the entire chromosome, or mitotic recombination.<sup>2-4</sup> While hundreds of TSGs have been identified, LOH studies typically focus on the TSG and/or closely flanking loci. For most TSGs, remarkably little is known about the extent and underlying mechanism(s) of LOH in tumor tissues.

In this study, we sought to examine the extent and mechanism of LOH in myeloid leukemias from children affected with neurofibromatosis 1 (NF1). The gene responsible for this disorder, *NFI* at chromosome band 17q11.2, encodes neurofibromin, a GTPase-activating protein that negatively regulates the biochemical activation of p21<sup>ras</sup> (Ras) family members (reviewed in<sup>5,6</sup>). Germline *NFI* mutations cause NF1, a dominant familial cancer syndrome that affects about 1 in 4000 persons. Clinical features of NF1 include neurocutaneous abnormalities, learning disabilities, and a predisposition to specific benign and malignant tumors (reviewed in<sup>7</sup>). Children with NF1 are at markedly increased risk of developing myeloid malignancies, particularly juvenile myelomonocytic leukemia (JMML).<sup>8</sup> JMML is an aggressive myeloproliferative disease (MPD) characterized by monocytosis, thrombocytopenia, splenomegaly, and by malignant infiltration of the skin, lymph nodes, lungs, liver and other organs (reviewed in<sup>9,10</sup>). Together, the biochemical activity of neurofibromin and

the dominant cancer predisposition seen in affected persons suggests that *NF1* functions as a TSG. Indeed, LOH at the *NF1* locus occurs in JMML and in other NF1-associated cancers (reviewed in<sup>11</sup>). Similarly, tumorigenesis in heterozygous *Nf1* mutant mice is associated with loss of the wild-type *Nf1* allele.<sup>12,13</sup> Consistent with the Knudson model of familial cancer genes, tumors from individuals with familial NF1 invariably show loss of the allele inherited from the unaffected parent (reviewed in<sup>11</sup>). Somatic intragenic *NF1* mutations have been identified in primary neoplasms, (reviewed in<sup>14</sup>), providing compelling evidence that functional inactivation of *NF1* is central to tumorigenesis. Deregulated Ras signaling has been reported in tumors from NF1 patients and *Nf1* mutant mice. These data are consistent with the idea that the tumor suppressor function of *NF1* is related to ability of neurofibromin to negatively regulate Ras output (reviewed in<sup>11,15,16</sup>).

In the course of investigating the extent and mechanism of LOH at the *NF1* locus in myeloid malignancies, we unexpectedly identified interstitial isodisomy for a large segment of chromosome 17 as a frequent underlying genetic mechanism. Remarkably, the LOH breakpoints clustered within centromeric and telomeric marker intervals in these leukemias. To our knowledge, this is the first report of interstitial isodisomy as a frequent mechanism of somatic TSG inactivation. These data have implications for uncovering novel TSGs and for understanding pathogenic mechanisms that contribute to the development of hematopoietic malignancies as well as solid tumors.

## **PATIENTS AND METHODS**

*Patients.* Clinical descriptions, LOH and mutation analyses of the *NF1* gene have been reported for most of the patients.<sup>17-19</sup> Selected demographic and laboratory data are summarized in Table 1.

Additional patient characteristics from previous reports are in Table S1. Study procedures involving human subjects were approved by the UCSF Committee for Human Research.

*NF1 Gene Dosage Assay.* This assay measures the copy number of *NF1* exon 32 by quantitating polymerase chain reaction (PCR) amplicons relative to those of a competitively-amplified disomic control locus. Validation of the assay on non-tumor DNA demonstrated that *NF1* disomy gave dosage values of  $0.98 \pm 0.08$  S.D., while monosomy gave values of  $0.45 \pm 0.04$  S.D. (S.D., one standard deviation). A range involving 2 S.D. was used to predict gene copy number (see Table 1 legend). Briefly, the assay is a quantitative, competitive PCR adapted from the method of Celi et al<sup>20</sup>. Assay conditions are available upon request.

*Fluorescence In Situ Hybridization (FISH).* Metaphase chromosome preparations of immortalized lymphoblastoid cells from patient 1 were prepared and hybridized as described previously.<sup>21</sup> The bacteriophage P1 probe P1-12, contains ~55 kb of sequence from *NF1* intron 27b.<sup>21</sup> BAC clone 1000G21 (17q25) was identified by hybridization of D17S928 amplicons to filter arrays of the RPCI-11 human male BAC library, segment 4 (Roswell Park Cancer Institute, Buffalo, NY). Hybridization signals were detected using a commercial system (Vector). Chromosomes were banded using Hoechst 33258-actinomycin D staining and counterstained with propidium-iodide and signals visualized by fluorescence microscopy using a dual-band pass filter (Omega).

Cryopreserved bone marrow samples were thawed and cultured at  $1 \times 10^6$  cells/ml for 24 hr (90% RPMI 1640/10% fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml Streptomycin, 10 mM HEPES) at 37°C in 95% air/5% CO<sub>2</sub>. Following incubation, the cells were exposed to hypotonic KCl (0.75 M, 8 min, 37°C), fixed in absolute methanol:glacial acetic acid (3:1), and air dried on slides. *NF1* probes were P1 bacteriophage clone P1-9, which spans ~65 kb of the *NF1* gene including exons 2-11, and clone P1-12.<sup>21</sup> Centromere-specific probes for chromosomes 7 and 17 (CEP®7-Spectrum Green™ and CEP®17-Spectrum Green™, Abbott Molecular, Inc, Abbott Park, IL), and the PAC clone P263P1 (Genome Systems Inc., St. Louis, MO), were hybridized as controls. P263P1 was isolated by screening

the PAC library using primers for *D5S479*, and contains an insert of 70 kb derived from 5q31. Labeled probes were prepared by nick-translation using Bio-11-dUTP (Enzo Diagnostics, New York, NY) or digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). Interphase FISH was performed as described previously.<sup>22</sup> Hybridization of probes labeled with either biotin or digoxigenin was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA) and rhodamine-conjugated anti-digoxigenin antibodies (Boehringer Mannheim), respectively. Nuclei were counterstained with 4,6,-diamidino-2-phenylindole-dihydrochloride (DAPI). The slides were randomized and examined by two observers in a blinded fashion, with 500 cells scored by each observer for each probe. We established control values by hybridizing the probes to cryopreserved bone marrow cells from patients in remission (C1, AML-M4) or with myeloid leukemias that retained heterozygosity at *NF1* (C2-C5 in Supplementary Table 2). The cut-off value was set as the mean $\pm$ 3 standard deviations.

The distribution of hybridization signals per nucleus for the CEP@17 probe was determined in bone marrow cells from healthy control individuals (N=10)(see Supplemental Table S2).

*Mapping the LOH Region.* Polymorphic loci were genotyped by PCR. LOH at the *NF1* locus was evaluated by PCR analysis of at least one informative intragenic site including exon 5, intron 27B *AluI/AluII*, and intron 38. LOH was determined by comparing the genotype of the patient's tumor DNA to that of peripheral blood DNA of the patient's parents. For patients 1, 4, 5, 9, and 10, normal tissue or an EBV-transformed cell line was available to confirm a constitutional genotype with biparental inheritance of *NF1* alleles.<sup>18,23</sup> Segregation of alleles from parents to child for multiple informative loci on autosomes other than 17 was consistent with parentage as stated for each case (data not shown). Physical distances between chromosome 17 loci are based on the May, 2004 assembly of the human genome (<http://genome.ucsc.edu>).

## RESULTS

*Delineation of a Large Region of Isodisomy in an NF1-Associated MPD.* Previous molecular analysis of bone marrow cells from children with NF1 revealed LOH at *NF1* in CD34<sup>+</sup> cells in three informative cases, whereas lymphoblasts immortalized by Epstein-Barr virus (EBV) retained heterozygosity in two of these patients.<sup>18</sup> The remaining child with LOH at *NF1* in EBV-transformed lymphoblasts was a 9-month-old boy with an unusual MPD and loss of the maternal *NF1* allele (Table 1, patient 1).<sup>18</sup> The retained paternal allele carried a *de novo* R1276X mutation (Table S1) that encoded a truncated protein lacking the GTPase activating protein (GAP) domain.<sup>19</sup> We performed extended LOH analyses of chromosome 17, which demonstrated loss of maternally-derived alleles from *D17S975* at 17q11.2 to *D17S1830* at 17q25.3, a segment of 50.3 Mb (Fig. 1). Although a deletion of this size is readily detected by cytogenetic techniques, the bone marrow and lymphoblastoid cells of patient 1 had a normal 46,XY karyotype. These data suggested that the 50.3 Mb LOH region was not deleted, but present on both chromosome 17 homologs. To address this possibility, the lymphoblastoid cells were analyzed by FISH with an *NF1* intron 27b probe.<sup>21</sup> Hybridization signals were detected on both chromosome 17 homologs (Fig. 2A). Together, these data suggest that a 50.3 Mb interstitial interval of the maternal chromosome had been replaced with a homologous paternal DNA segment, resulting in homozygosity for the mutant R1276X *NF1* allele (Fig. 1). FISH with a BAC harboring the *D17S928* locus, confirmed that the heterozygous 17qter segment had not translocated elsewhere in the genome of the leukemic clone (Fig 2B). Taken together, these data confirmed interstitial isodisomy.

*Uniparental Isodisomy is a Frequent Mechanism of LOH in NF1-Associated Leukemias.* The unexpected findings in patient 1 prompted us to use a quantitative *NF1* gene dosage PCR assay to assess *NF1* copy number in 9 additional NF1-associated leukemia specimens with LOH at *NF1*.<sup>17-19</sup>

Bone marrow DNA from patient 1 (Table 1) and from the normal tissues of his parents (data not shown) gave *NF1* gene dosage values ranging from 0.91 - 1.08, which are consistent with disomy. Surprisingly, the *NF1* dosage values for 7 of the 9 remaining NF1-associated myeloid malignancies were also consistent with disomy, whereas two cases had values consistent with monosomy (Table 1). Cryopreserved bone marrow specimens were available from 5 of these patients for copy number confirmation. FISH analyses using a chromosome 17 centromere-specific probe (Cep®17) and two *NF1* probes (P1-9 and P1-12) provided physical confirmation that the leukemias of all four cases in which the dosage assay predicted disomy contained two *NF1* alleles (Fig. 2D; patients 3-6 in Supplemental Data Table S2). By contrast, the bone marrow of patient 10 demonstrated monosomy for *NF1* with two signals in 67% and one signal in 29% of cells (Fig. 2D and Supplemental Data, Table S2), which was also consistent with the gene dosage assay (Table 1). To confirm that the cells being examined were from the malignant clone with LOH at *NF1*, a chromosome 7-specific probe (Cep®7) was hybridized to bone marrow cells of patient 3, who had monosomy 7 and was disomic at *NF1* as measured by both gene dosage and FISH (Table 1, Figure 2D, Supplemental Data Table S2). As expected, dual-color FISH revealed monosomy 7 in cells that also had two structural *NF1* alleles (Figs. 2C, 2D, and Supplemental Data Table S2). Together these studies demonstrate that LOH at *NF1* in myeloid malignancies is preferentially associated with isodisomy of a chromosomal segment carrying the mutant *NF1* allele.

*Clustering and Parental Origin of Chromosome 17 LOH Breakpoints.* Each of the 8 leukemias with isodisomy at *NF1* showed a large segment of LOH that minimally ranged from 50-52.7 Mb (Fig. 1). Among these cases, both the proximal and distal LOH breakpoints were clustered. In all 8, the centromeric breakpoints mapped to a maximum interval of 4.9 Mb (6% of the chromosome 17 length

of 78.77 Mb) between *D17S959* and *D17S1294*. In some leukemias, additional informative markers narrowed the breakpoint interval, as in patient 5 with a 2 Mb interval between *D17S1878* and *D17S975*. With the exception of case 6, the minimum common breakpoint region is 2 Mb and is delineated by *D17S33* and *D17S975*. The distal breakpoints in patients 1, 2, 3, and 6 were clustered between *D17S1830* and *D17S928*, an interval of 2.4 Mb (Fig. 1). Lack of informativeness at *D17S928* and other 17qter loci tested precluded determining if the large LOH segments in the tumors of patients 4, 5, 7, and 8 were isodisomic.

The bone marrows of patients 9 and 10 had LOH at loci flanking the *NF1* region. The leukemias of these 2 patients showed structural deletions that included the *NF1* locus as determined by gene dosage (Table 1) and FISH data (Figure 3D, Supplemental data Table S2). The centromeric breakpoints mapped to a 1.2 Mb interval between *D17S1294* and *NF1* intron 38 and the telomeric breakpoints were in a 7.5 Mb interval between *D17S1800* and *D17S250*. These data are consistent with an interstitial deletion ranging from 268 kb (*NF1* ex38 to *D17S1800*) to 9 Mb (*D17S1294* to *D17S250*).

The bone marrows of patients 1,2, 3, and 8 lost chromosome 17 maternal alleles and were isodisomic for a paternally-derived DNA segment, whereas the cells of patients 4-7 lost paternal alleles and were isodisomic for a maternally-derived interval of comparable length (Fig. 1). In each patient with familial NF1, the isodisomic segment was derived from the parent with NF1 (Tables 1 and S1). The bone marrow of patient 10, who had *de novo* NF1, showed loss of the maternal *NF1* allele (Table 1 and Fig. 1B). These data infer that patient 10 carried a germline mutation of the paternal *NF1* allele and underwent somatic deletion of the normal maternal *NF1* allele, which is consistent with the reported parental predisposition for *NF1* germline mutations.<sup>24,25</sup>

## DISCUSSION

Our analyses of myeloid malignancies from children with NF1 uncovered two distinct mechanisms underlying inactivation of the normal *NF1* allele: interstitial isodisomy and interstitial deletion (Fig. 3A). The somatic interstitial deletions involving *NF1* are of interest as they may occur by a mechanism similar to that identified in constitutional and somatic mosaic *NF1* microdeletions in normal (nontumor) tissues. In these cases, the deletions are 1.2-1.4 Mb and occur by non-allelic homologous recombination between pairs of high identity low-copy number repeat (LCR) elements that flank the *NF1* gene (reviewed in<sup>26</sup>). Microdeletions mediated by germline or somatic recombination between different LCR pairs<sup>27,28</sup> both involve the entire *NF1* gene and the D17S1800 locus, which are deleted in the tumors of patients 9 and 10. A mechanism of LCR-mediated recombination could be tested by precise mapping of the deletion breakpoints by single nucleotide polymorphism (SNP) mapping in the myeloid malignancies. Alternatively, the length of these somatic microdeletions in myeloid malignancies may be constrained if a 50% reduction in the expression of a critical flanking gene(s) inhibited outgrowth of the leukemic clone.

While segmental or interstitial uniparental isodisomy has been reported in constitutional rearrangements (reviewed in<sup>29</sup>), we were surprised to find that interstitial isodisomy for 50-52.7 Mb of chromosome 17 is a common mechanism underlying LOH in NF1-associated myeloid malignancies. The leukemias of each of the four patients (nos.1, 2, 3, and 6 in Fig. 1) in whom *D17S928* was informative had interstitial isodisomy. The remaining four tumors (nos. 4, 5, 7, 8 in Fig. 1) may also be interstitial, but *D17S928* or other regional markers were not informative. We propose that interstitial isodisomy results from a double mitotic recombination event between chromatids of the two chromosome 17 homologs during the S/G2 phase of the cell cycle of a leukemia-initiating cell (Fig. 3B). Depending upon the segregation pattern during mitosis, the daughter cells would have biparental

inheritance at all chromosome 17 loci, or alternatively, would show interstitial isodisomy (Fig 3B). Only one of four possible daughter cells would have interstitial isodisomy along with homozygous inactivation of *NFI*, the latter of which is presumably essential for leukemic outgrowth.<sup>30,31</sup> The low frequency of double mitotic recombination events, estimated at about  $10^{-10}$  in normal lymphocytes<sup>32</sup>, infers that *NFI* inactivation confers a strong proliferative advantage in the leukemia-initiating cell. A possible mechanism may involve non-allelic mitotic homologous recombination between LCRs or Alu elements, which has been implicated in recurring translocations, isochromosomes, deletions and amplifications in tumor tissues.<sup>33-35</sup> Other mechanisms that could give rise to 17q interstitial isodisomy are less likely. For example, two sequential single recombination events in different precursor cells are also possible, but would imply that each independent event conferred a proliferative advantage. A gene conversion-like event of a 50 Mb segment would be unprecedented as estimated conversion tracts in humans are typically <2 kb.<sup>36,37</sup> The clustered breakpoint intervals of the isodisomic segments suggests that mitotic recombination may be favored in these regions.

Our data are intriguing in light of recent reports showing *JAK2* point mutations in most patients with polycythemia vera (PV) and in some cases of essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF).<sup>38-41</sup> An unexpected and intriguing result of these studies was the finding of biallelic mutations in ~30% of the PV specimens. The underlying genetic mechanism in these cases was a mitotic recombination that led to loss of the normal *JAK2* allele and resulted in isodisomy for a segment of the short arm of chromosome 9 estimated to span ~40 cM.<sup>42</sup> The most telomeric marker studied showed LOH in many cases, which is consistent with a single recombination event. We did not prove that the isodisomic regions were interstitial in patients 4, 5, 7, and 8 due to a lack of informative polymorphic markers near 17qter (Figure 1), and it is therefore possible that isodisomy resulted from a single recombination event in one or more of our cases. Similarly, it is

possible that studies with additional markers near 9pter would uncover double mitotic recombination events in some PV samples. Whereas biallelic *NF1* inactivation deregulates Ras signaling in response to hematopoietic growth factors, it is less obvious why loss of the normal *JAK2* allele and isodisomy of the mutant homolog would confer a growth advantage beyond that of a dominant heterozygous mutation. Since JAK2 molecules that are recruited to activated growth factor receptors trans-phosphorylate each other, it is possible that the normal protein has a dominant interfering activity that impairs the ability of mutant JAK2 to deregulate downstream effectors. Consistent with this idea, James et al<sup>38</sup> found that co-expressing wild-type and mutant JAK2 proteins restored erythropoietin-dependence in the Ba/F3 pro-B cell line.

A broad implication of our work and of the recent studies of *JAK2* mutations in MPD is that segmental uniparental isodisomy may be a frequent but unrecognized mechanism in human cancers. Although isodisomy of a 25 cM interval was first described in child with Down syndrome who developed acute lymphoblastic leukemia by Rogan *et al.*<sup>43</sup>, this genetic mechanism has received limited attention in hematologic malignancies until recently. The availability of automated allelotyping and the use of SNP and high-density arrays have been developed for high-resolution analysis of allelic losses and gains in tumors.<sup>44-46</sup> Interestingly, a number of investigators are now identifying regions of isodisomy in acute myeloid leukemia<sup>47-49</sup>. Our data extend these studies by showing that inactivation of a known myeloid TSG is frequently associated with acquired uniparental disomy. Importantly, DNA segments that are associated with partial or interstitial isodisomy will appear normal when examined by conventional cytogenetic analysis, FISH, or comparative genomic hybridization, making these approaches of limited use for cancers where LOH results in isodisomy. Together, LOH and copy number analyses provide the opportunity to define new genetic mechanisms of somatic mutation, mitotic recombination sites, putative modifying or imprinted genes, and/or correlations between tumor

genotype and neoplastic transformation. In addition, mono- or bi-allelic expression from a locus (loci), other than the TSG itself, could affect the efficacy of putative therapeutic agents.

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Table 1. NF1 gene dosage in bone marrows of children with NF1 and malignant myeloid disorders.

Patient No.	Sex	Age at Onset	Diagnosis	Parental Origin		NF1 Gene Dosage Value <sup>a,b</sup>	Predicted NF1 Gene Copy No. <sup>c</sup>
				NF1 mutation	LOH at NF1 Locus		
1	M	9 mo	MPS	paternal	maternal	0.99	disomy
2	M	10 mo	AML	paternal	maternal	0.93	disomy
3	M	24 mo	monosomy 7	unknown	maternal	0.96	disomy
4	M	14 mo	JMML	maternal	paternal	0.86	disomy
5	F	30 mo	JMML	maternal	paternal	0.94	disomy
6	M	10 mo	JMML	maternal	paternal	0.87	disomy
7	M	5 mo	monosomy 7	maternal	paternal	0.90	disomy
8	F	18 mo	MPS	paternal	maternal <sup>a</sup>	0.85	disomy
9	M	5 yr	JMML	maternal	paternal	0.57	monosomy
10	M	19 mo	monosomy 7	<i>de novo</i>	maternal <sup>a</sup>	0.48	monosomy

Clinical descriptions, LOH and NF1 mutation analyses have been reported previously for most of the patients.<sup>17-19</sup>

<sup>a</sup> Data from this study employing quantitative PCR at NF1 gene segment.

<sup>b</sup> Measured in unfractionated bone marrow cells, except patients 3 and 9 for whom leukemic cells in peripheral blood were used.

<sup>c</sup> Predicted gene copy number based on NF1 gene dosage values. Validation of the assay on non-tumor DNA demonstrated that NF1 disomy gave dosage values of  $0.98 \pm 0.08$  S.D., while monosomy gave values of  $0.45 \pm 0.04$  S.D. (S.D., one standard deviation). A range of 2 S.D. was used to approximate a 95% confidence interval. Therefore, values from 0.82 - 1.14 predicted disomy and values from 0.37 - 0.53 predicted monosomy.

## FIGURES AND LEGENDS

Figure 1. LOH at chromosome 17 loci in NF1-associated myeloid malignancies. Ideogram and schematic of chromosome 17 showing the loci that were screened for LOH. For each tumor, a bar shows the single chromosome 17 that underwent LOH with informative loci (tic marks), segments showing biparental inheritance (white), segments that underwent LOH (black), segments where a recombination event occurred (grey), and qter segments that lacked informative loci (?). Below the schematic, the parental origin is given for the isodisomic and deleted regions for each tumor. For patients 1-3, and 8, the maternal homolog is shown with the region of paternal isodisomy indicated in black. For patients 4-7, the paternal homolog is shown with the region of maternal isodisomy indicated in black. LOH in patients 9 and 10 occurred by intrachromosomal deletion indicated in black. Additional chromosome 17 loci that were tested, but not informative, are not shown. Physical distances (rounded to the nearest tenth of Mb) are based on the May 2004 assembly of the human genome (<http://genome.uscs.edu>), in which the length of chromosome 17 is given as 78,774,742 bp.

Figure 2. Fluorescence in situ hybridization analysis of NF1-associated myeloid malignancies. Panels A and B show metaphase spreads of EBV-transformed cells from patient 1 that were hybridized with *NF1* probe P1-12 (panel A) and BAC clone 1000G21, which contains the *DI7S928* locus at 17q25 (panel B). Each of 20 metaphase cells examined showed signals on both chromosome 17 homologs, consistent with disomy. The chromosome 17 homologs were identified by Hoechst/actinomycin D staining, which reveals a Q-banding like pattern. Panel C shows dual-color FISH performed by co-hybridizing a digoxigenin-labeled probe P1-12 (Rhodamine signal) and an  $\alpha$ -satellite probe specific for the centromere of chromosome 7 (CEP®7, SpectrumGreen), which showed monosomy 7 and *NF1* disomy in bone marrow cells from patient 3. Panel D is a graphic summary of interphase FISH

analyses of myeloid leukemia cells with *NFI* (red triangle) and control probes. Probe 263P1 is a 70 kb PAC clone containing *D5S479* (chromosome band 5q31) (yellow squares). CEP®17 is a centromere-specific probe for chromosome 17 (green circles) and CEP®7 is a centromere-specific probe for chromosome 7 (blue diamond). C1 is a cryopreserved bone marrow sample from a patient with AML-M4 in complete remission. Control samples C2-C5 are cryopreserved bone marrow samples from 4 children with myeloid leukemias that retained heterozygosity at the *NFI* locus. The mean distribution of signals for the chromosome 17 centromere-specific probe was determined by the interphase analysis of bone marrow cells from 10 healthy individuals. This graph is a summary of data given in Supplementary Data Table 2.

Figure 3. LOH in *NFI*-associated myeloid malignancies and proposed mechanism of interstitial isodisomy. The schematic in panel A depicts the two different patterns of LOH observed in the tumors. The inactivated *NFI* allele (*NF*<sup>-</sup>) is marked with an X on the chromosome, while the normal *NFI* allele (*NF*<sup>+</sup>) is indicated by diagonal hashmarks (///). The interstitial isodisomic and deleted regions can be of maternal or paternal in origin. Panel B. Proposed mechanism for double mitotic recombination during the S/G2 phase of the cell cycle leading to interstitial uniparental isodisomy in a leukemic-initiating cell. The four possible daughter cells are depicted, along with their *NFI* genotypes and disomy patterns. Although this example depicts a cell with maternal interstitial isodisomy and *NFI* inactivation, paternal interstitial isodisomy was also observed in our study (Fig.1).

Figure 1.

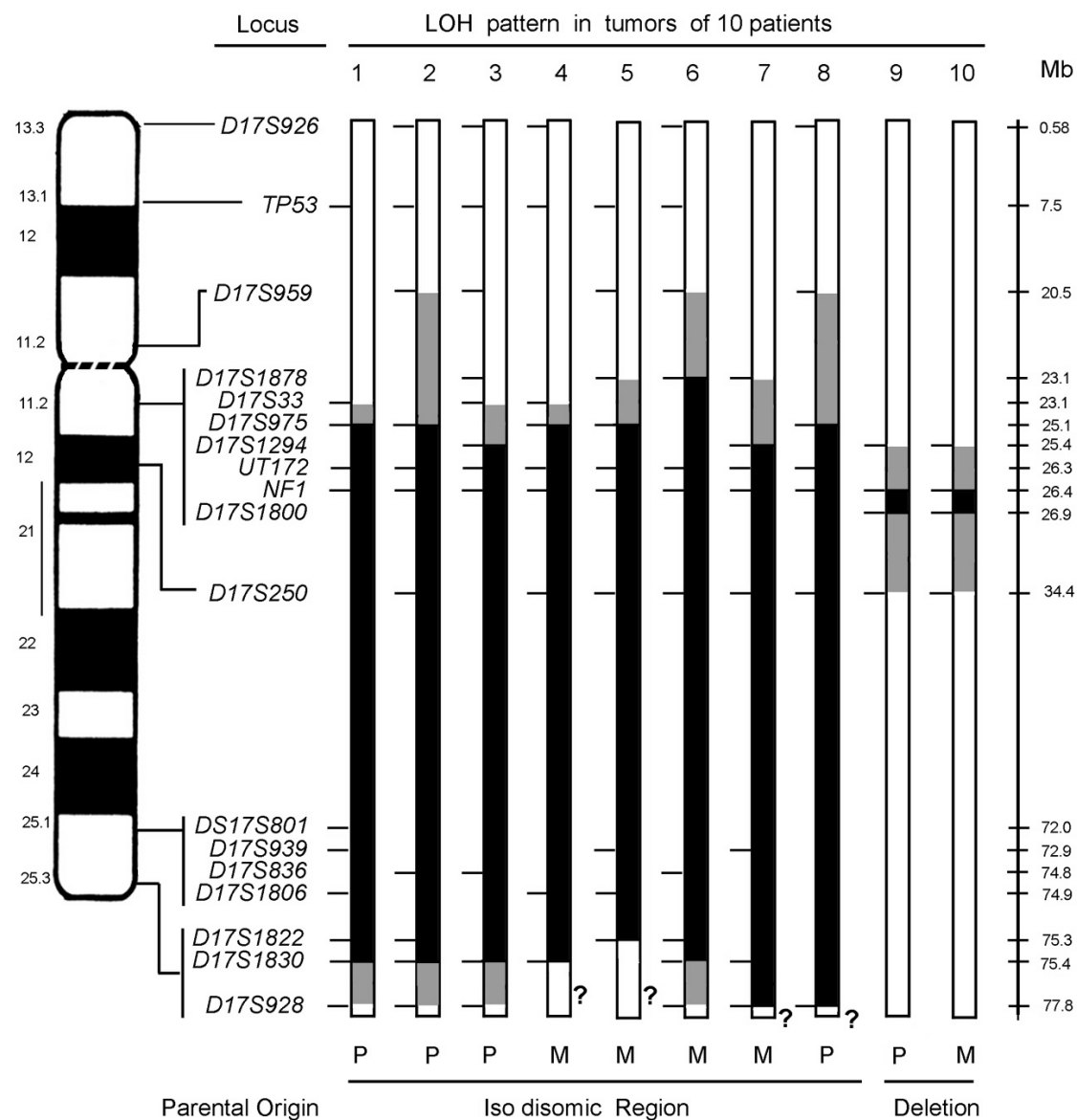
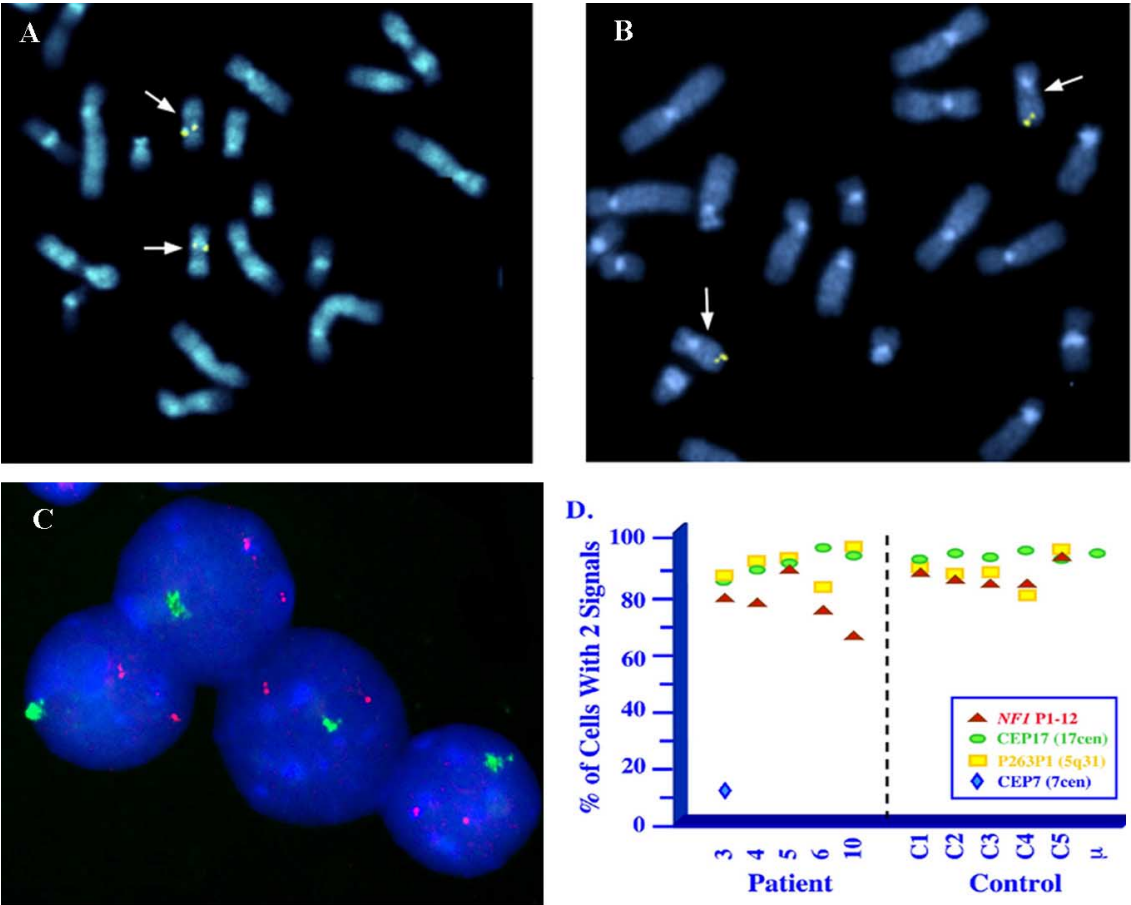
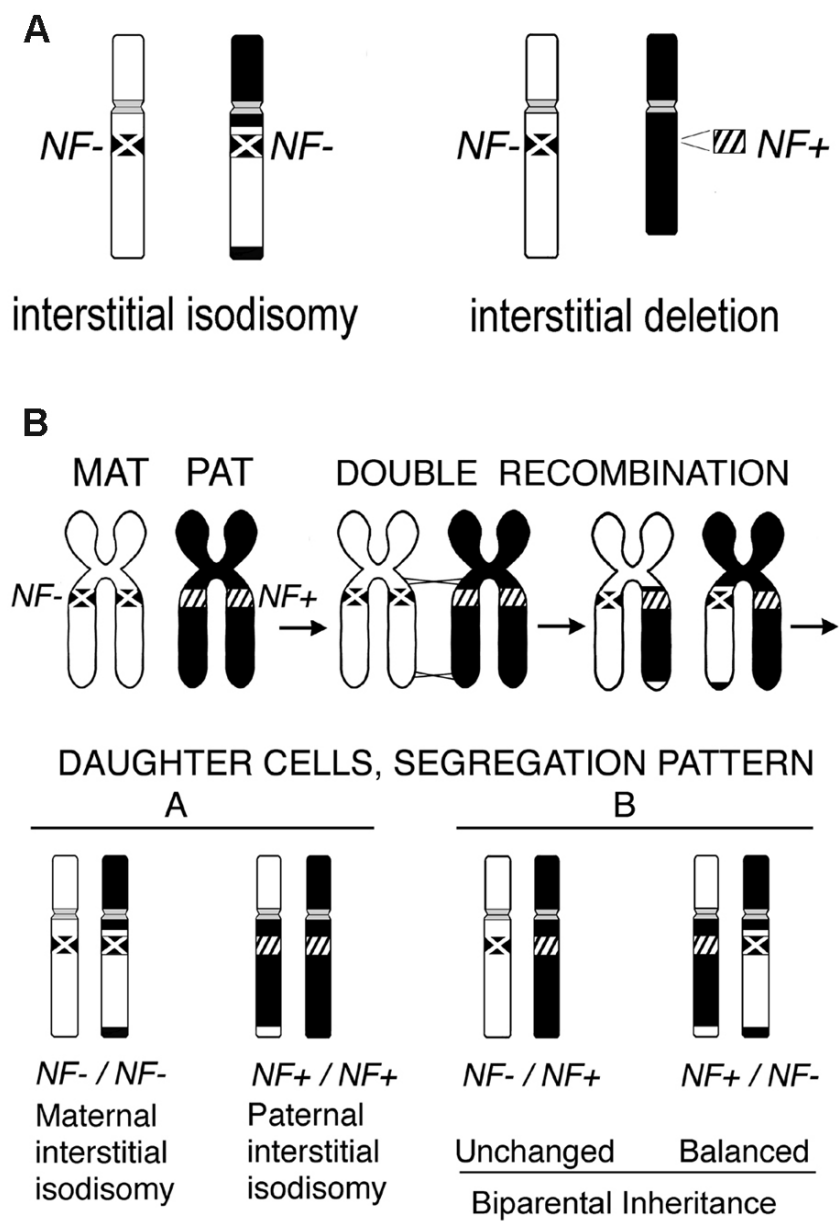


Figure 2.



**Figure 3.**



## Supplementary Tables and Figures

Table S1. Selected Characteristics and Previous Reports of Patients Included in This Study

Case #	Uniparental Disomy	Parent with NF1	<i>NF1</i> Mutation	Case Number in Previous Publications
1	yes	Father	C to T at nt 3826 (R1276X)	#8 in Ref. <sup>17</sup> ; #4 in Ref. <sup>19</sup>
2	yes	father	4914delCTCT	#2 in Ref. <sup>17</sup> ; #5 in Ref. <sup>19</sup>
3	yes	unknown	unknown	#1 in Ref. <sup>18</sup>
4	yes	mother	C to T at nt 4538	#3 in Ref. <sup>18</sup> ; #9 in Ref. <sup>19</sup>
5	yes	mother	unknown	#8 in Ref. <sup>18</sup>
6	yes	mother	unknown	#1 in Refs <sup>17</sup> and <sup>19</sup>
7	yes	mother	intron 36, splice donor	#6 in Ref. <sup>17</sup> ; #2 in Ref. <sup>19</sup>
8	yes	father	unknown	#11 in Ref. <sup>17</sup> ; #8 in Ref. <sup>19</sup>
9	no	mother	5024delT	#7 in Ref. <sup>18</sup> ; #11 in Ref. <sup>19</sup>
10	no	neither	Intron 34, splice donor	#6 in Ref. <sup>11</sup>

**Table S2. Interphase Fluorescence in Situ Hybridization Analysis of Bone Marrow Samples.**

Patient* No.	Chr. 17† Probes	Number of Hybridization Signals (Percent per 1000 nuclei)					Control† Probes	Number of Hybridization Signals (Percent per 1000 nuclei)				
		0	1	2	3	≥4		0	1	2	3	≥4
3	CEP <sup>R</sup> 17	0	6	87	3	4	CEP <sup>R</sup> 7	0	88	12	0	0
	P1-9	0.6	14	84	1	0.4	263P1	0	7.6	90	2	0.4
	P1-12	1	13	80	6	0	263P1	0	9	88	2	1
4	CEP <sup>R</sup> 17	0	5	90	2	3						
	P1-9	0.5	10	86	3	0.5	263P1	0	12.5	84	2	1.5
	P1-12	0.5	13	78	8	0.5	263P1	0.4	7	92	0.6	0
5	CEP <sup>R</sup> 17	0	6	93	0	1						
	P1-9	6	7	82	4	1	263P1	1	4	90	4	1
	P1-12	0.4	6	90	2.6	1	263P1	0.2	4.8	93	0.5	1.5
6	CEP <sup>R</sup> 17	0	2	97	1	0						
	P1-9	1	5	95	0	0	263P1	0	25	74	1	0
	P1-12	1	9	76	12	2	263P1	0	15	84	0	1
10	CEP <sup>R</sup> 17	0.8	3.6	95	0.6	0						
	P1-12	4	29	67	0	0	263P1	0	3	97	0	0

**Table S2. Continued.**

Patient* No.	Chr. 17† Probes	Number of Hybridization Signals (Percent per 1000 nuclei)					Control† Probes	Number of Hybridization Signals (Percent per 1000 nuclei)				
		0	1	2	3	≥4		0	1	2	3	≥4
C1	CEP <sup>R</sup> 17	0	6.4	93	0.6	0						
	P1-9	3	7	89	1	0	263P1	2	3	86	5	4
	P1-12	2	5	89	2	2	263P1	0	7	90	1.5	1.5
C2	CEP <sup>R</sup> 17	1	3	95	1	0						
	P1-9	2	22	76	0	0	263P1	1	19	79	1	0
	P1-12	0.5	7	86	5	1.5	263P1	1.6	6	88	3	1.4
C3	CEP <sup>R</sup> 17	0	5	94	1	0						
	P1-9	1.5	12	81	4	1.5	263P1	0	8	90	2	0
	P1-12	0	9	85	3.4	2.6	263P1	0	8	89	2	1
C4	CEP <sup>R</sup> 17	0	4	96	0	0						
	P1-9	0.5	11	83	1.5	2	263P1	0.5	9	88	2	0.5
	P1-12	0.5	9.5	85	4	1	263P1	0.5	17	82	0.5	0
C5	CEP <sup>R</sup> 17	0	6	93	1	0						
	P1-9	1	6	92	0.5	0.5	263P1	0.6	4	95	0.2	0.2
	P1-12	0	5	94	1	0	263P1	0	3	96	0.8	0.2
Control‡ N=10	CEP <sup>R</sup> 17											
	Mean	0.15	4.5	95	0.24	0.15						
	S.D.	0.19	0.79	0.83	0.25	0.16						

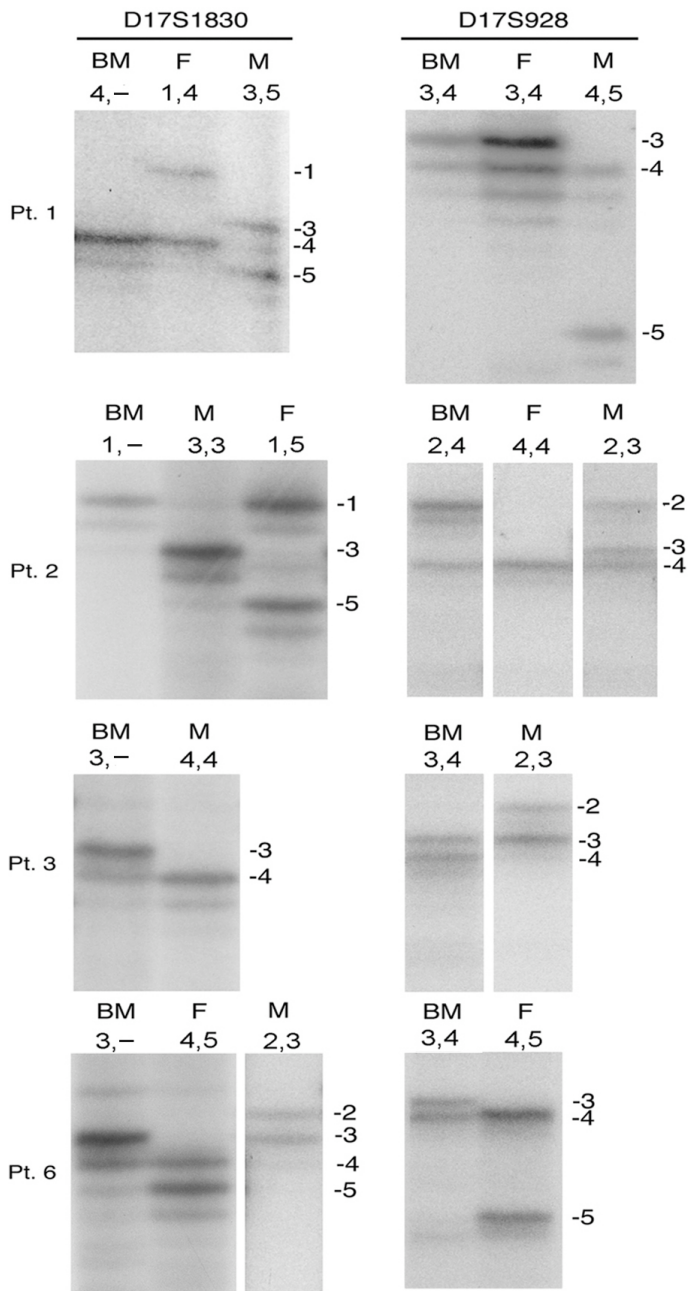
- \* Patients 3-6 and 10 are described in Table 1. C1 is a cryopreserved bone marrow sample from a patient with AML-M4 in complete remission. C2-C5 are cryopreserved bone marrow samples from 4 children with myeloid leukemias that retained heterozygosity at the *NFI* locus.
- † Probe 263P1 is a 70 kb PAC clone containing D5S479 (5q31). CEP<sup>R</sup>17 is a centromere-specific probe for chromosome 17. CEP<sup>R</sup>7 is a centromere-specific probe for chromosome 7. Due to the nature of the samples analyzed by FISH, i.e., cryopreserved bone marrow samples, we established control values by hybridizing the probes to cryopreserved bone marrow samples (C1-C5). Using the standard methodology for FISH studies, the cut-off value was established as the mean $\pm$ 3 SD. The cut-off values for one signal were: CEP<sup>R</sup>17, 6.9%; 263P1, 24%; P1-9, 28.6%; P1-12, 12.8%. In only 2 cases did the test value exceed the cut-off value. In the first case, the percentage of cells with only 1 signal for the *NFI* P1-12 probe observed for Patient 10 (29%) exceeded the cut-off value of 12.8%. Due to limited material from Patient 10, it was not possible to perform additional studies. In the second case, the percentage of cells with one signal for the PAC 263P1 (5q31) observed for Patient 6 (25%) exceeded the cut-off value for this probe (24%); however, the percentage of cells with one signal was 15% in a second hybridization of this probe, well below the cut-off value.
- ¶ The distribution of signals for the chromosome 17 centromere-specific probe was determined by the interphase analysis of bone marrow cells from 10 healthy individuals.

### **Supplementary Figure Legends**

Figure S1. LOH at chromosome 17 loci in leukemic cells. Selected examples of LOH analysis at loci in the tumors of patient 1, 2,3, and 6 are shown. For patient 1, the maternal allele of D17S1830 was lost, while both parental alleles were retained at D17S928. Similarly, patient 2 showed LOH for a maternal allele at D17S1830 and retention of both alleles at D17S928. The bone marrow of patients 3 and 6 lost heterozygosity at D17S1830, but retained heterozygosity at D17S928. DNA sources were BM, patient bone marrow; F, Father's leukocytes; M, mother's leukocytes. The genotype of each individual is given above the lane; patient genotypes are shown as having only one allele, as these data only document LOH not isodisomy.

Figure S2. Sequence of D17S928 in bone marrow of Patient 1. To confirm heterozygosity of D17S928 in Patient 1, the locus was amplified and sequenced directly. D17S928 is a dinucleotide repeat and heterozygosity at this locus is confirmed by the frame shift in the sequence at position 60 in this figure.

Supplementary Figure S1



Supplementary Figure S2

